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Simplified Low Copy Number Dna Analysis By Post Pcr Purification

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SIMPLIFIED LOW COPY NUMBER DNA ANALYSIS BY POST PCR PURIFICATION

by

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ABSTRACT

Frequently evidentiary items contain an insufficient quantity of DNA to obtain complete or even partial DNA profiles using standard forensic gentotyping techniques. Here, various methods of post PCR purification were evaluated for their effects on the sensitivity of fluophore-based allelic detection. A method of post PCR purification is described which increases the sensitivity of standard 28 cycle PCR such that low copy number DNA templates (<100 pg DNA) can be analyzed. Full profiles were obtained with as little as 20 pg template DNA without increased cycle number. In mock case type samples with dermal ridge fingerprints, genetic profiles were obtained by amplification with 28 cycles followed by post-PCR purification whereas no profiles were obtained without purification of the PCR product. Allele drop-out, increased stutter, and contamination (allele drop-in) typical of LCN analysis were observed. A single incident of contamination was observed in a reagent blank (not duplicated upon re-amplification) however, no contamination was observed in negative amplification controls.

To my husband, Mike, and my children Michael, Elizabeth, and Allison whose love and support sustain me. To my Father and Mother, Richard and Jean Fowler, to whom I will always be eternally grateful, and to my Lord and savior Jesus Christ who guides, strengthens and enlightens me.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS/ABBREVIATIONS

LCN	Low Copy Number
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate

CHAPTER ONE: INTRODUCTION

Recently a great deal of interest has been generated in obtaining DNA profiles from low template DNA samples including DNA transferred by casual contact, often referred to as trace DNA. Wickenheiser et.al. have demonstrated that profiles can be obtained from fingerprints and other objects that have been handled (1, 9, 16, 19, 21). However, success rates using standard forensic procedures are relatively low ranging from 30% to 50% (21). Studies aimed at increasing sensitivity through extract concentration and reduced volume PCR have been performed with a measure of success (5, 13). With current standard forensic procedure the limit of detection for a DNA profile is anywhere from 100 pg to 500 pg dependent upon the amplification multiplex and detection parameters utilized (23, 24).

Samples containing <100 pg DNA fall into a category where specialized low copy number (LCN) techniques are employed. The most popular method of LCN analysis is to raise the number of amplification cycles from 28 to 30-34 cycles (1, 6, 7, 9, 12, 16, 18, 20). Other strategies such as nested PCR and whole genome amplification have also been described (6, 8). These methods have proved highly successful in obtaining profiles from as little as 5 pg of DNA. However low copy number analysis is not without its drawbacks. Typical problems encountered are allelic drop-out and drop-in, higher stutter peaks, and sporadic contamination. Not withstanding these problems, strategies for dealing

with these issues have been described (6, 7, 20) and LCN analysis sits at the forefront of forensic DNA inquiry.

An alternative method of LCN analysis through post PCR purification is set forth in this study. In the forensic community STRs are typically examined with a multiplex amplification procedure and detected via capillary electrophoresis. Prior to electrophoresis the sample is electrokineticaly injected into the capillary, typically for 5 seconds. During electrokinetic injection a voltage is applied to the electrode, effectively drawing negatively charged molecules such as DNA into the capillary. The short injection time permits a limited amount of sample to be taken into the capillary. This process is known as sample stacking. Sample staking occurs when the ionic strength of the sample is lower than the ionic strength of the buffer in the capillary. As the voltage is applied (usually 15,000 V) resistance and field strength increase at the mouth of the capillary due to the presence of fewer ions in the sample to carry the current. This causes the sample ions to migrate rapidly to the capillary. As the sample ions enter the polymer solution in the capillary their movement slows and the sample stacks in a sharp band. In this process the uptake of smaller components is favored; STR amplicons compete with primers, unincorporated dNTPs, salts and other negatively charged PCR reaction components. In theory, the removal of left over amplification components should favor amplicon injection and lead to an increase in fluorescent signal intensity (Figure 1).
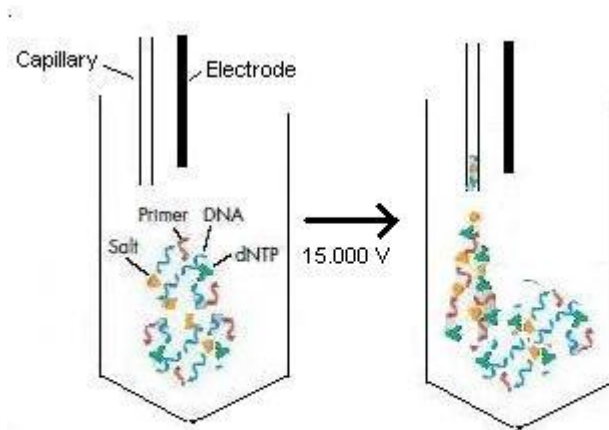
2

Figure 1: Electrokinetic injection. Competition from salts, primer and dNTPs (above). Amplicon injection favored after purification (below).

This study explores the effects of increasing PCR sensitivity without increased amplification cycles. Four post PCR purification products incorporating

three purification methodologies are examined to determine their efficacy purifying

the PCR product in an attempt to increase fluorescent allelic signal intensity.

CHAPTER TWO: LITERATURE REVIEW

Low Copy Number Analysis

Papers reporting the PCR amplification of low template quantities of DNA down to the single cell level have been published since the late 1980's (26). However, the application of a technique to the forensic sciences requires validation to ascertain its reliability and limitations (17). Problems associated with low copy number (LCN) analysis such as allelic drop-out, allelic drop-in, increased stutter and contamination produce interpretation issues that have hindered its embrace by the forensic community (22, 25). The introduction of a set of interpretational guidelines set forth by Gill (6, 7) appears to have increased interest in LCN analysis.

The most commonly reported LCN technique is the use of increased amplification cycles. Gill (7) varied amplification cycles from 28 to 56 cycles and studied its effect on the number of alleles observed, heterozygote imbalance and stutter. Gill concluded that 34 cycles was the optimum and obtained full profiles down to 25-50 pg. At 34 cycles Gill observed increased heterozygote imbalance and increased stutter noting that a new set of interpretational guidelines would be required for low copy number analysis. Whitaker (20) amplified 12 pg and 25 pg DNA using 34 cycles and compared the profile characteristics to 1ng DNA amplified with 28 cycles (current standard analysis procedure). Whitaker observed

the characteristics of allele drop-out, heterozygote imbalance, and stutter concluding all three characteristics were increased with 34 cycle amplification. Whitaker reported the mean stutter proportion for 12 pg and 25 pg DNA samples to be within the range of 1 ng DNA samples, however, outlier data recorded stutter proportions up to 40%. This falls well above the accepted maximum stutter value of 15% for standard PCR amplifications. Surprisingly, Kloosterman (12) reported no increase in stutter using 34 cycle PCR, but did observe heterozygote imbalance.

In addition to increased cycle PCR, nested PCR and whole genome amplification strategies have been employed. Gill (7) added a nested PCR regime to his study and found evidence to suggest using nested singleplex primers would produce more alleles, however issues with increased stutter and heterozygote allele imbalance remain. Hanson (8) employed whole genome amplification (WGA) strategies, pre-amplifying genomic template to overcome the stochastic effects of LCN amplification. Amazingly, using a modified improved primer extension pre-amplification technique full profiles were obtained from autosomal and Y-STR multiplexes using 5 pg of input DNA. The autosomal multiplex was altered to 32 a cycle amplification using a reaction volume of 12.5 µL in this experiment. Heterozygote imbalance was not improved by this process ranging from 15-95%. Issues with stutter were not reported. Caragine, Gill, Hansen, and Kloosterman (2, 7, 8, 12) all reported allelic drop-in or non-specific amplification during low copy number analysis.

The characteristics of LCN analysis such as allelic drop-out, increased stutter and allelic drop-in create difficulties interpreting LCN profiles with single source and mixed stains. Gill (6, 7) set forth guidelines for LCN amplification and profile interpretation. He adopted the recommendation of Taberlet (22) for replicate analysis where an allele was only reported if observed twice in the replicate amplifications. A likelihood ratio method that assessed DNA profiles in the light of sporadic contaminants, stutter and allelic drop-out was described by Gill. Gill demonstrated that the duplicate analysis method was conservative in relation to likelihood ratios as long as sporadic contamination was < 30% per locus. Gill also addressed the role of negative controls recommending their function as a "health check" of the process, but noting that alleles found in negative controls not corresponding to sample alleles are inconsequential.

Reduced volume PCR has also been explored as a method for obtaining additional allelic information. This technique relies on standard 28 cycle amplification. Gaines (5) showed that small amounts of DNA amplified in reduced volume reactions yielded the same concentration of amplified product as proportionally increased reaction volumes. Therefore 0.2 ng DNA amplified in a 5 µL reaction volume yields the same PCR product concentration as 2 ng DNA amplified in a 50 µL reaction volume. During the sensitivity experiments of this study 0.03 ng DNA amplified in a 10 µL reaction volume identified 32.4% of the sample alleles at an analysis threshold of 50 RFU. No incidences of contamination or allelic drop-in were reported, but heterozygote imbalance occurred. Leclair (13)

7

also studied the effects of reduced volume PCR on signal intensity and heterozygote balance, concluding no significant imbalance with 10 µL reactions down to the amplification of 0.125 ng, but increased imbalance with 5 µL reactions.

Comparing the sensitivities of LCN techniques can be problematic as amplification conditions, multiplexes, and instrument platforms vary from study to study. In addition, according to Kline (11) in a NIST study on quantitation accuracy and its effect on multiplex signal intensity, variability is associated with specific instruments reagents and analysts. The variability in instrument sensitivity was confirmed by Krenke (23) who reported up to a 5 fold difference in instrument sensitivity among 19 different ABI PRISM® 310 Genetic Analyzers.

PCR Purification

Purification of the PCR product is routinely used for mtDNA analysis prior to cycle sequencing. In 1994 Mezei (14) examined purification of the PCR product for the removal of primers, primer-dimers, Taq DNA polymerase, salts, and dNTP's. He compared purification by filtration (Amicon® Centricon™ microconcentrators) to silica resin purification (Promega Wizard™ PCR Preps system) examining the retention of ssDNA (29-73 bp) and dsDNA (50-1500 bp).

Recoveries for ssDNA were as follows:

ssDNA	Silica	Filtration
73 bp	1.1%	28.4%
45 bp	1.5%	3.2%
29 bp	1.0%	7.6%

While recoveries for dsDNA 500 bp and less are listed below:

dsDNA	Silica	Filtration
500 bp	98.4%	70.2%
300 bp	99.1%	66.7%
200 bp	68.6%	63.8%
100 bp	8.1%	39.3%
75 bp	3.2%	25.3%
50 bp	1.9%	18.7%

He concluded that the silica based system did a superior job removing primers and noted the nearly 100% recovery of 300 bp and greater PCR product. This 1994 publication did not focus on the 100-400 bp range that is currently the target of most forensic STR amplifications.

More recently Dugan (4) compared four purification products for DNA recovery, ease of use, and quality of sequence profiles. Dugan examined filtration (Microcon-100), two silica columns (Concert Rapid PCR Purification columns and Qiagen QIAquick PCR purification columns), and enzyme mediated hydrolysis (ExoSAP-IT®). For recovery of DNA the Qiagen and ExoSAP-IT® methods

performed best with 75% and 78% recoveries respectively. The Microcon-100 averaged a 30% DNA recovery. ExoSAP-IT® was recommended for its ease of use.

Interestingly, Hutchinson (10) purified microsatellite PCR products from collard lizards that were analyzed on a slab gel. He reported a dramatic reduction in background and lane bending resulting in an improvement in the signal to noise ratio of two to five. As a side note he purified a triplex of microsatellite product from two individuals. Purified and unpurified aliquots were subjected to capillary electrophoresis resulting in a 3.5 fold increase in fluorescent signal intensity. Other unpublished studies (2, 15) have reported significant increases in fluorescent signal intensities using capillary electrophoresis to detect microsatellites after using post PCR purification.

LCN Samples

DNA profiles are sometimes obtained from touched objects without employing specialized low copy number techniques. Oorschot (19) obtained DNA profiles from swabs of handled objects. His studies indicated that a substantial transfer of DNA occurred upon first contact and that extended handling time did not significantly increase the amount of DNA transferred. Also tested were objects handled by two or three individuals. The profile of the last holder was usually

observed with the previous holders showing up in the mixture to varying extents. He cautioned that the predominant profile obtained was not always that of the last holder. Wickenheiser (21) confirmed that DNA transfer was independent of handling time, and reported its dependence upon the individual handler and the handled substrate. He defined individuals as sloughers and non-sloughers of epithelial cells, and referred to such transfers as trace DNA. Success rates for obtaining genetic information using standard techniques ranges from 30-50% according to Wickenheiser.

With the utilization of 34 cycle PCR Findlay (25) reported a 91% success rate obtaining genetic information from single cells and a 50% success rate obtaining a full STR profile (6 loci). Schulz (16) analyzed fingerprints lifted with tape and swabbed from glass surfaces. A 30 cycle singleplex PCR amplification was employed from 10 µL of extract. Results were obtained from 24% of the samples which included fingerprints processed with soot and magnetic powder. Balogh (1) amplified fingerprints from paper using 38 cycle PCR and reported correctly typed profiles 80-93% of the time. Also reported was a decrease in the success rate to 47% when fingerprints had been treated with enhancement chemicals (ninhydrin, iodine and soot powder). Other authors (9, 18) have reported genetic information from telogen hair roots (34 cycle, singleplex) and semen samples from vasectomized males (30 cycle, 6 plex Y-STR's).

While genetic information from low copy number samples is becoming more and more promising, Gill (6) et. al. warn of certain dangers assessing the

11

relevance of such evidence. For example, minute or degraded bloodstains may yield genetic information, but associating the profile with the bloodstain is problematic, as the source could be underlying cells in the substrate. Likewise, a profile obtained from a fingerprint may be from the underlying substrate or secondary transfer from the fingerprint processing materials.

CHAPTER THREE: METHODOLOGY

Isolation and Quantitation of DNA

DNA was isolated from two different, previously typed blood samples ('CTS" and "LH') on cotton cloth and FTA paper using a standard organic extraction method and quantified using the Quantiblot Kit (Applied Biosystems, Foster City, CA). DNA for the sensitivity studies was prepared from serial dilutions of the 1.25 ng/µL 'CTS' and 0.625 ng/µL 'LH' samples.

Amplification

The DNA was amplified using the AmpFℓSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems) in a reaction volume of 25 µL. Amplification was performed in a GeneAmp® PCR 9700 thermocycler (Applied Biosystems) for a 95°C 11 min incubation followed by 28 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min, ending with a 60°C for 60 min extension and 25°C hold in accordance with the manufacturers recommendations.

PCR Purification

Filtration

Filtration membranes allow the passage of smaller substances through the membrane while retaining the sample, which is then reconstituted off the membrane. Forensic STR amplicons range from approximately 100 bp to 400 bp in length while primers, the largest of molecules competing during electrokinetic injection, are typically around 20 bp in length. Amplified product was purified using the Microcon-50 and Montage PCR® filter units. The Microcon-50 filter is a low binding regenerated cellulose membrane designed to retain > 90% of double stranded DNA 100 bp or larger. Single stranded DNA has a > 90% retention at 125 bp or larger (30). The Montage PCR® filter utilizes a patent pending size exclusion technology and is optimized for PCR product $\geq$ 100 base pairs boasting a > 99% primer removal rate (29). This straightforward process is illustrated below in Figure 2.
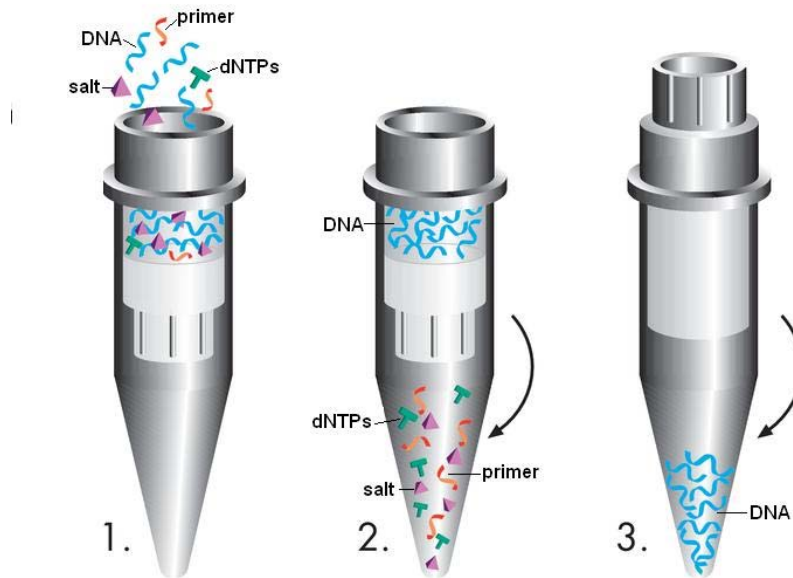
Figure courtesy of Millipore.com

Figure 2: Filtration methodology used by Microcon-50 and Montage PCR® filter units.

Filtration was performed by adding 375 µL TE^{-4} Buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 8) to the sample reservoir followed by 25 µL of the amplified product. The tubes were subjected to centrifugation (maximum speed for 12 min for the Microcon-50 and 1,000 x g for 15 min for the Montage PCR®). After discarding the eluate, 400 µL of TE buffer were added to the sample reservoir followed by a brief vortex and centrifugation as described above. This process was repeated for a total of four washes. The sample reservoir was placed in a clean collection tube and 10 µL of TE^{-4} Buffer were added to the reservoir followed by a brief vortex,

inversion of the sample reservoir and centrifugation (1,000 x *g* for 3 min for the Microcon-50 and 1, 000 x g for 2 min for the Montage PCR®).

Silica Gel Membrane

Silica-gel membranes bind DNA in high chaotropic salt conditions (27). Nucleic acids are believed to be adsorbed to the silica-gel when high concentrations of chaotropic salts form a cation bridge between the silica and the phosphate backbone of DNA (28) (Figure 3). Impurities are washed away in an ethanol containing buffer and the purified DNA eluted in a low salt buffer or water.
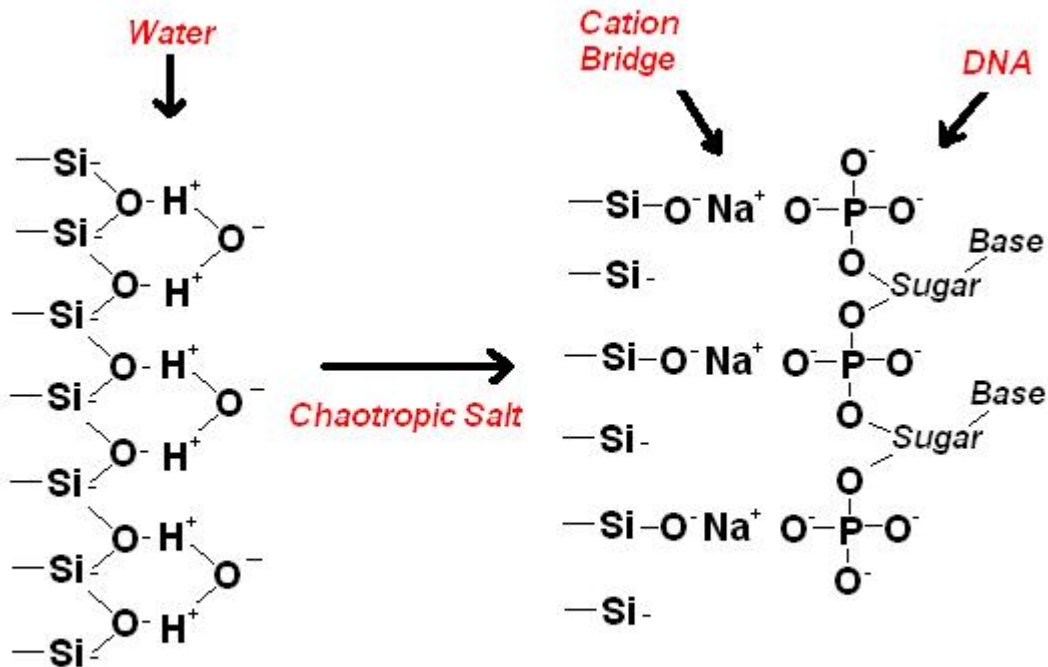
Figure 3: Proposed mechanism for nucleic acid binding to silica.

Purification with the Qiagen MinElute Kit was performed by adding 125 µL high salt, low pH PB buffer to the column followed by 25 µL of amplified product and centrifugation at ≥ 10,000 x g for 1 min. After discarding the eluate, 700 µL of ethanol containing PE buffer were added to the column to wash and centrifuged at ≥ 10,000 x g for 1 min. The eluate was discarded and this step was repeated for a total of 4 washes followed by a dry centrifugation under the same conditions to

clear the column. The amplified product was eluted with 10 µL low salt, high pH

EB buffer into a clean 1.5 mL tube. The precise volume of eluate for the Microcon-

50, Montage PCR® and MinElute purifications was measured in order to normalize

for variability in volume recovery.

Enzyme Mediated Hydrolysis of Reaction Components

ExoSAP-IT® reagent utilizes the hydrolytic enzymes Exonuclease I and

Shrimp Alkaline Phosphatase (SAP) to remove unwanted dNTPs and primers. It is

used routinely to deactivate reaction components prior to down stream applications

such as cycle sequencing and SNP analysis (31). Exonuclease I degrades single

stranded DNA in the 3´ to 5´ direction producing 5´mononucleotides and a terminal

5´-dinucleotide, as long as the 3´ terminus is not phosphorylated (32) (Figure 4).

Shrimp Alkaline Phosphatase dephosphorylates the 5´ ends of dNTPs rendering

them inactive in down stream applications (33) (Figure 5).

18
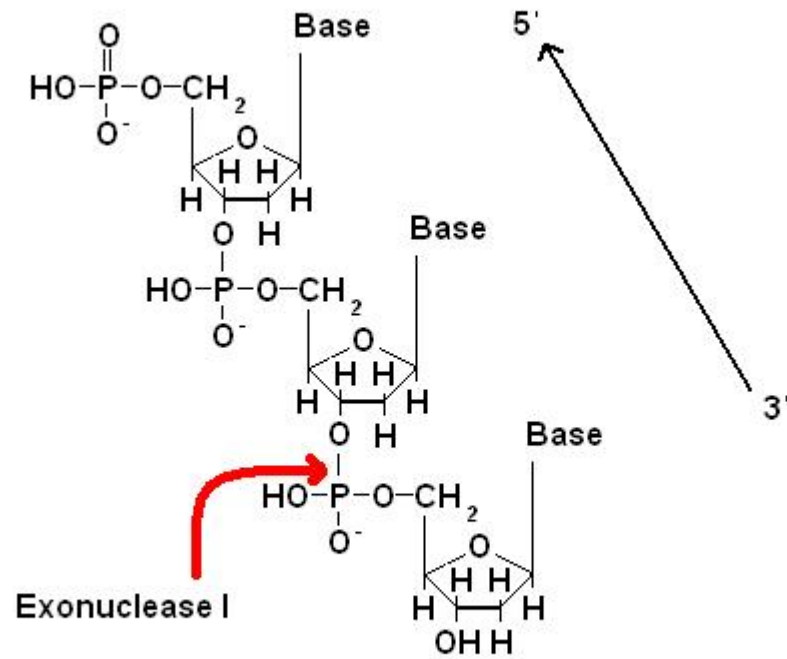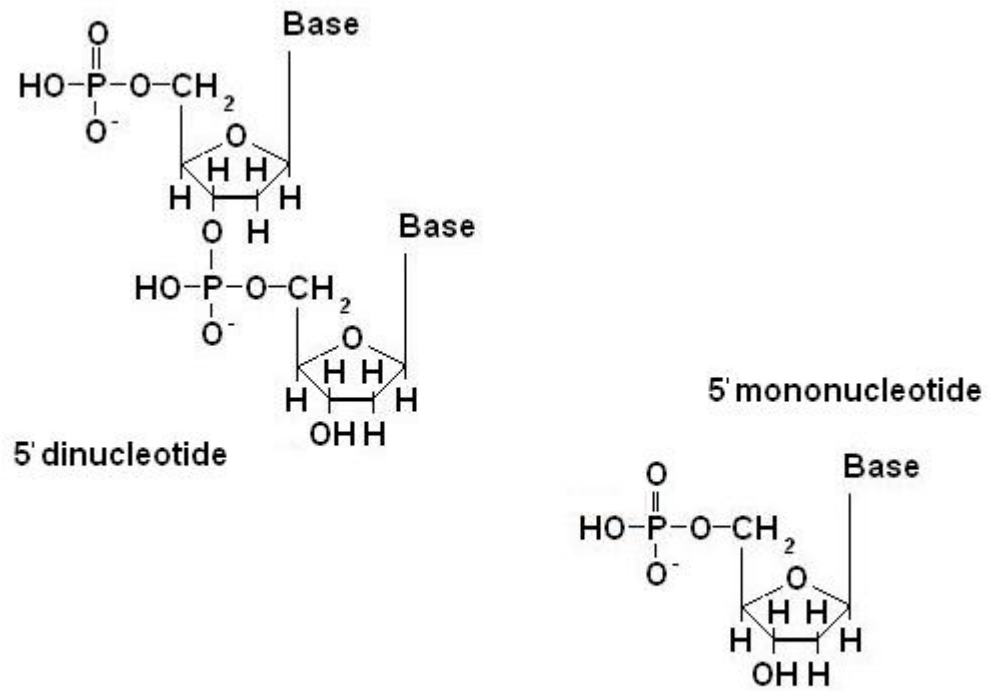
Figure 4: Exonuclease I activity on single stranded DNA
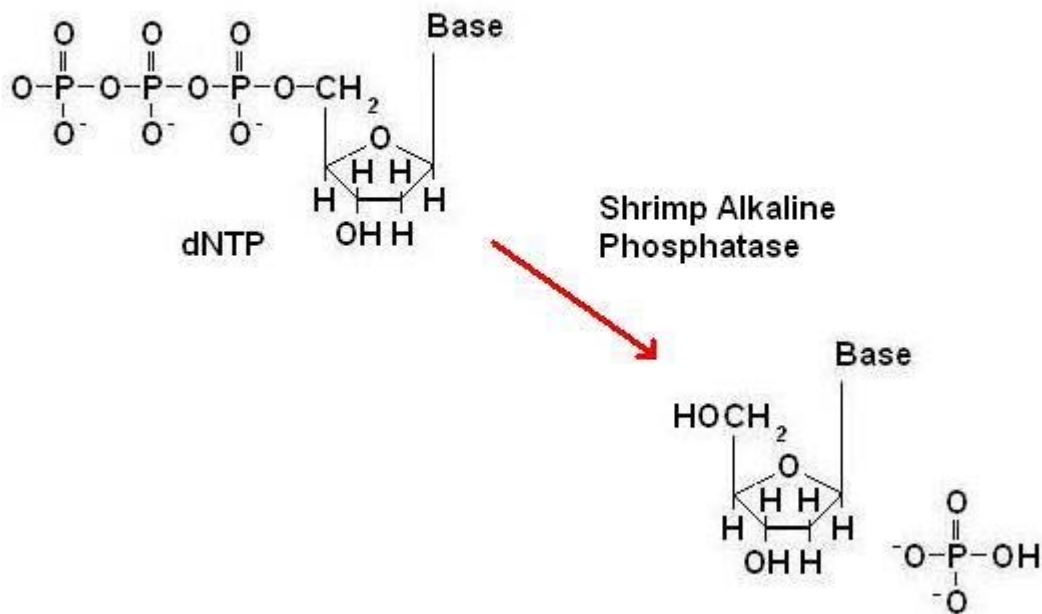
Figure 5: Shrimp Alkaline Phosphatase activity on dNTPs.

ExoSAP-IT® reagent was added to amplified product in a ratio of 2 µL ExoSAP-IT® to 5 µL PCR product as recommended by the manufacturer. The entire 25 µL of amplified product was treated and incubated in a GenAmp® PCR 9700 thermocycler at 37°C for 15 min followed by heat inactivation of the enzymes at 80°C for 15 min. The enzyme-treated amplified product was stored at -20°C until use.

Separation and Detection of STR Alleles

Samples were prepared for electrophoresis by adding 1.5 µL of unpurified PCR product with 0.5 µL GeneScan-500 LIZ Size Standard and 24.5 µL Hi-Di™ Formamide unless other wise specified. Purified amplified product was prepared using 1.5 µL PCR product, 0.1 µL GeneScan-500 LIZ Size Standard and 25µL Hi-Di™ Formamide unless other wise specified. The samples were heated to 95°C for 3 min and snap-cooled for at least 3 min. PCR products were separated and detected on the ABI Prism® 310 Genetic Analyzer using POP-4™ polymer (Applied Biosystems) with the GS STR POP4 (1mL) G5 module. The data was analyzed with GeneMapper 3.2 NT software (Applied Biosystems) using a threshold of 100 RFU.

Comparison of Post-PCR Purification Methods

The four purification methods were evaluated by a comparison of profile integrity and relative fluorescent signal intensity. The 1.25 ng/µL DNA extract was diluted to permit the amplification of 156 pg, 78 pg, 39 pg, and 20 pg total input template DNA. The diluted samples, negative amplification control, and reagent blank were amplified in quadruplicate. The samples were injected in triplicate to establish an average RFU for each allele before purification. Each input quantity of DNA along with the negative amplification control and reagent blank was subjected

to a different PCR purification procedure and injected in triplicate. The elution volume for the Microcon-50, Montage PCR®, and MinElute purification methods was 10 µL. The average peak height (PH, measured in RFU) for each allele, after purification, was calculated and then normalized to account for variation in eluate volume. The fold increase (FI) in signal intensity for each allele was calculated by $FI = PH_{purified}/PH_{unpurified.}$ The average fold increase across all alleles for each sample was determined. Peak heights for unpurified samples were obtained by analysis at 20 RFU.

Efficacy of Post PCR Purification Using Silica Gel Membrane (MinElute)

A direct comparison was made between unpurified and purified PCR product using the MinElute method. The 1.25 ng/µL and 0.625 ng/µL DNA extracts were diluted to permit the amplification of 625 pg, 312 pg, 156 pg, 78 pg, 39 pg, 20 pg, 10 pg, and 5 pg DNA. The samples were amplified in duplicate using a 25 µL reaction volume. 1.5 µL of the unpurified amplified product were added to the formamide mix and injected under standard conditions. The samples were again amplified in duplicate and purified with the MinElute method eluting into 25 µL. The samples were prepared for electrophoresis by adding 1.5 µL of the purified product with 0.1 µL LIZ size standard to the formamide mix and injected. The negative amplification control and reagent blank were also purified and injected.

The fold increase in signal intensity for each allele was calculated and the average fold increase for each input quantity of DNA was determined.

Use of Concentrated Purified, Total PCR Product

The effects of injecting the entire MinElute purified product were investigated. Serial dilutions of the 1.25 ng/µL and 0.625 ng/µL extracts were prepared such that 5-625 pg DNA was amplified in duplicate and concentrated to 10 µL in a heat assisted rotary evaporator. The 10 µL of unpurified PCR product was added to 15 µL of formamide and 1.5 µL LIZ size standard and injected. The same samples were amplified in duplicate with 5-78 pg DNA, post PCR purified using the MinElute system and eluted into 10 µL of EB buffer. The 10 µL of purified PCR product were added to 15µL formamide and 0.2 µL LIZ size standard and injected. The negative amplification control and reagent blank were similarly purified and injected.

Non-Probative Case Type Samples

To assess the effectiveness of PCR purification on casework type samples two dermal ridge fingerprints (on paper and glass substrates) and a telogen hair root were collected from three subjects. The fingerprint on paper was collected on a sheet of commercial printing paper taken from the center of a ream. The subjects

were asked to briefly hold a 2 cm X 5 cm piece of paper. Microscope slides were removed from the center of a new package and cleansed with ethanol. The subjects were asked to briefly hold the slide with the thumb on top. The slides were swabbed on the top with sterile water and the swabs were dried. The hairs were washed in sterile distilled water prior to extraction. The samples were extracted with the Qiagen mini blood extraction kit and concentrated to a volume of 25 µL. Quantification was performed with the Quantifiler quantification kit (Applied Biosystems). The samples were amplified in duplicate with the Identifiler™ kit under standard amplification conditions.

CHAPTER FOUR: RESULTS

Comparison of Post PCR Purification Methods

Four post-PCR purification methods were evaluated initially by a comparison of profile integrity and relative fluorescent signal intensity. These included two filtration methods (using the Millipore Corporation Microcon-50 and Montage PCR® filters), binding to a silica gel membrane (Qiagen MinElute PCR Purification Kit) and removal of primers and nucleotides by hydrolytic enzymes (ExoSAP-IT® from USB). DNA samples (156 pg, 78 pg, 39 pg, and 20 pg) were amplified in quadruplicate. The samples were injected in triplicate to establish an average RFU for each allele before purification. Each input quantity of DNA along with the negative amplification control and reagent blank was subjected to a different PCR purification procedure and injected in triplicate. The elution volume for the Microcon-50, Montage PCR®, and MinElute purification methods was 10 µL. The average fold increase across all alleles for each sample was determined.

Increase in allelic signal intensity compared to the standard non-post-PCR purified product was observed with the Microcon-50 filter (3-6 fold), the Montage PCR® filter (6-8 fold), and with the MinElute silica column (4-6 fold) (Figure 6).

26
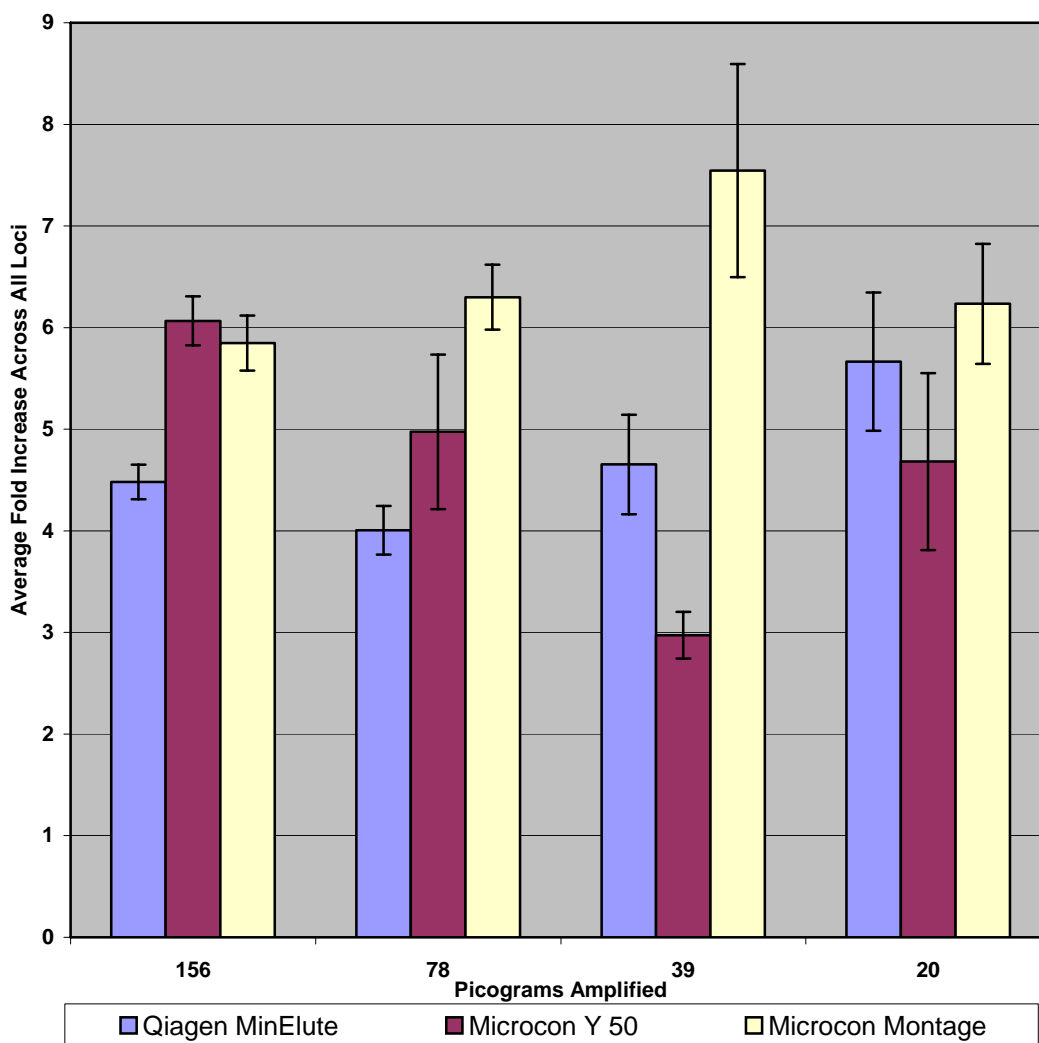
Figure 6: Comparison of Qiagen MinElute, Microcon-50, and Microcon Montage PCR® purification products. Normalized data represents 1.5µL of concentrated purified product (10µL eluate) in formamide mix.

The ExoSAP-IT® results yielded poorer quality data, exhibiting a decrease in RFU, minus A, extraneous peaks and substantial quantities of 75-100 bp products (Figure 8).

Taq polymerase has the characteristic of adding an additional nucleotide to the 3' end of the amplicon. This non-template addition is encouraged during PCR by the addition of a 60 min 60 °C extension cycle. This addition is typically adenosine and is therefore referred to as the +A form. Incomplete adenylation, or in this case lose of the adenylated form through exonuclease activity produces split peaks or shoulder peaks as illustrated in Figure 7.
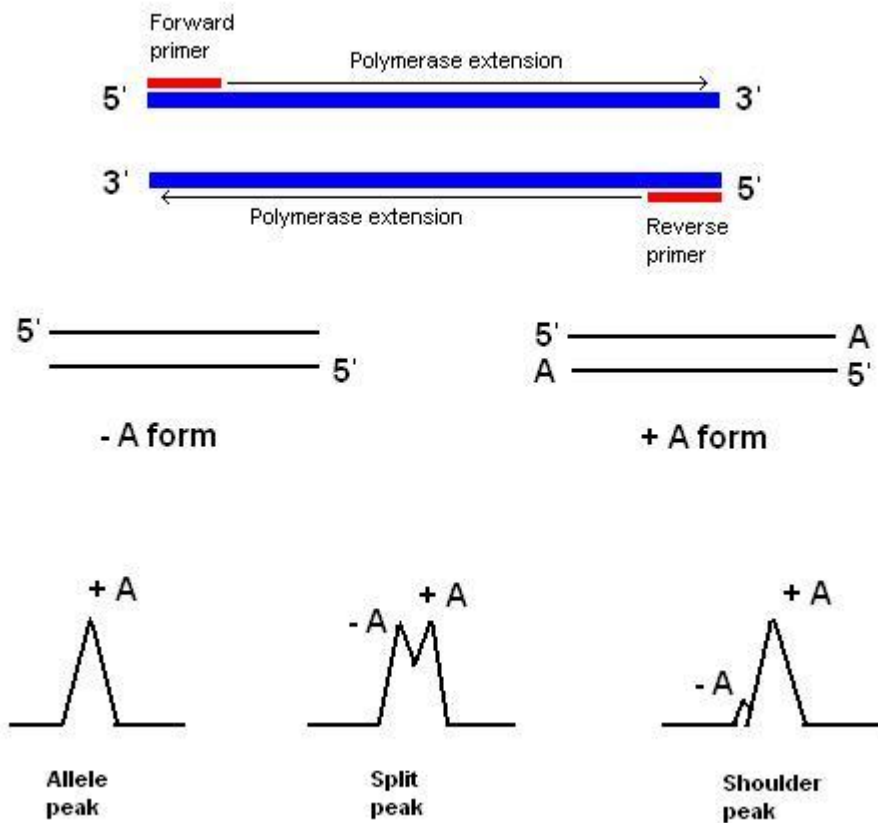
Figure 7: Minus A. Exonuclease activity results in the lose of the adenylated form creating split peaks and shoulder peaks.

Since ExoSAP-IT® treatment degrades reaction components and does not remove them, ExoSAP-IT® treated samples were further purified using the MinElute column to remove excess degraded reaction components and injected. This eliminated most of the anomalies seen except for the minus A as can be seen in Figure 9. ExoSAP-IT® treatment followed by MinElute purification did an excellent job removing the residual primers (Figure 10) and would be a superior technique if the minus A issue could be resolved.
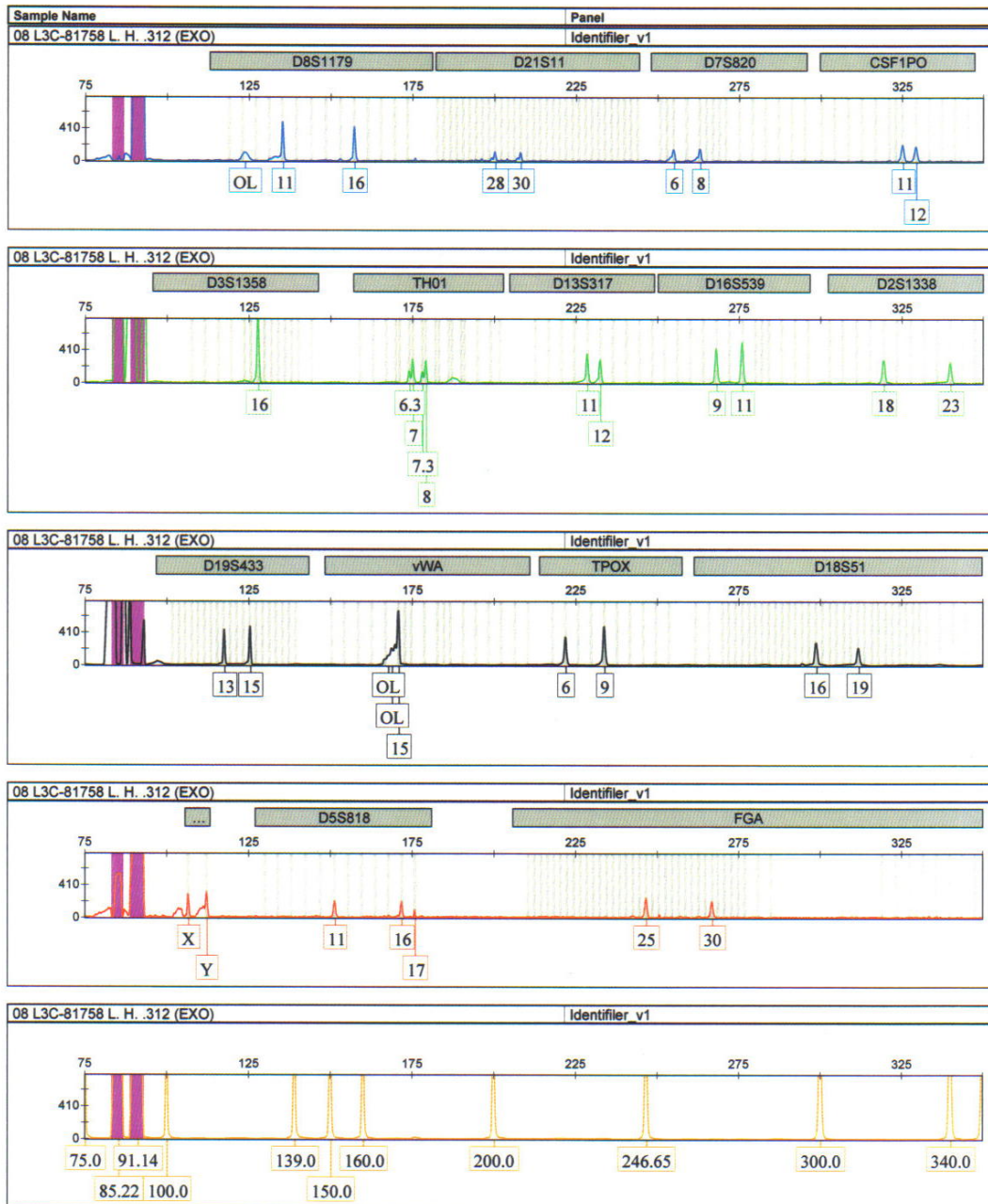
Figure 8: Amplification of 312 pg treated with ExoSAP-IT®. 1.5 µL of the ExoSAP-IT® treated PCR product was injected.
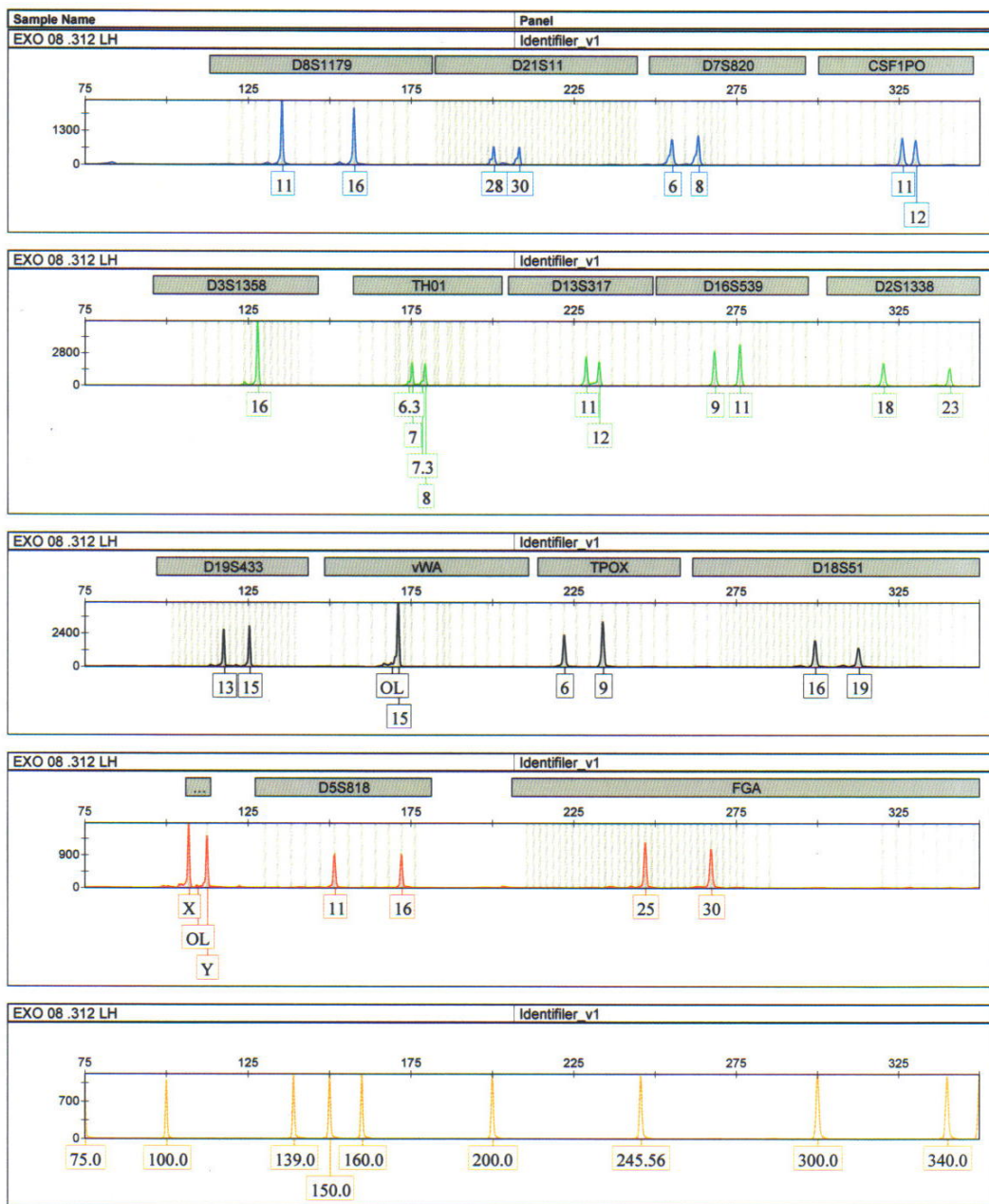
Figure 9: Same ExoSAP-IT® treated sample as figure 2 further purified and concentrated with MinElute (10 μL eluate) with 1.5 μL injected.
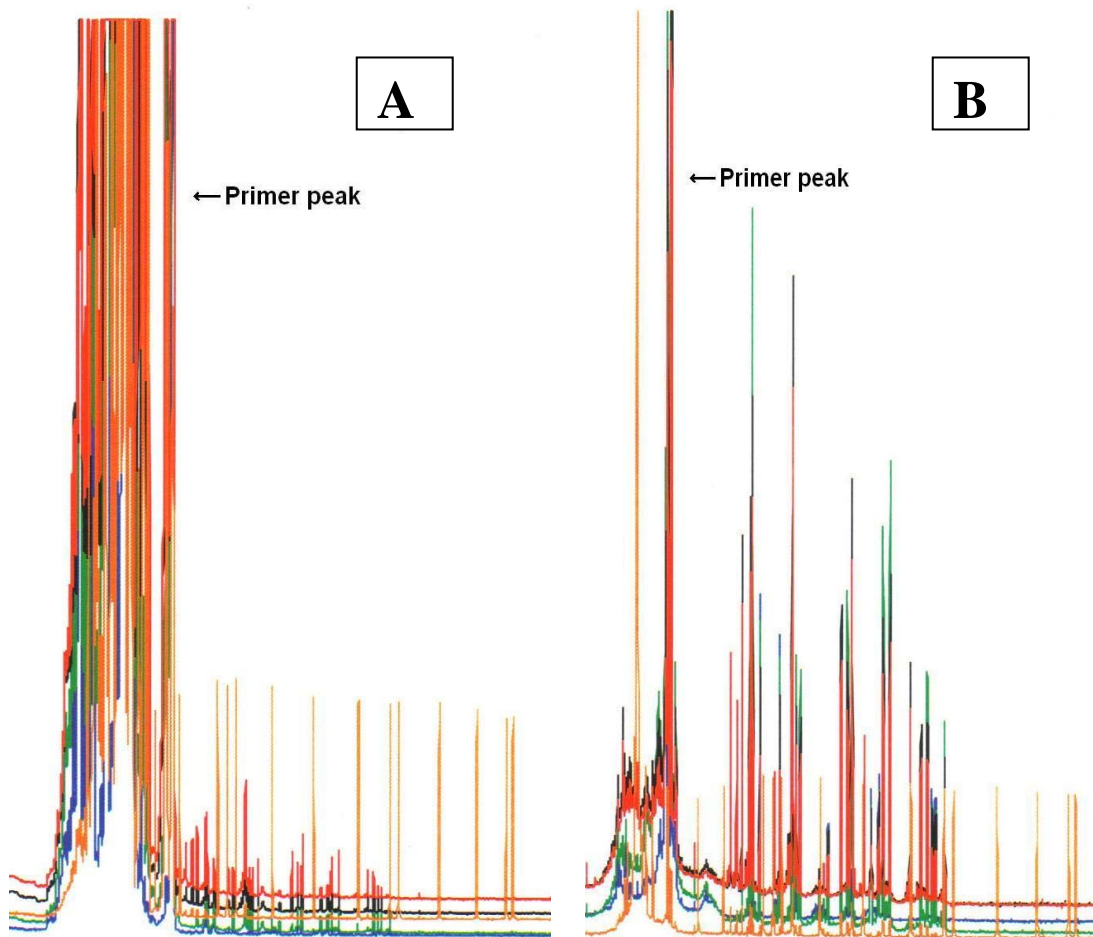
Figure 10: Primer peak of ExoSAP-IT treated sample (A). Primer peak of same ExoSAP-IT treated sample after MinElute purification (B).

Non-specific product resulting in off ladder calls was observed at D3S1358 using both filtration methods. Off ladder alleles were called by the software in 11 out of 14 injections using the Microcon-50 and in 4 out of 14 injections with the

Montage PCR® filter. These artifacts were observed below threshold in samples that did not possess off ladder alleles. In contrast, the MinElute purified samples exhibited no off ladder calls at D3S1358 and little artifact below threshold (Figure 11). Though the Montage PCR® filter yielded a greater signal increase, the MinElute method was selected for subsequent studies due to the absence of artifacts and its ease of use (1 min centrifugation as opposed to 15 min).
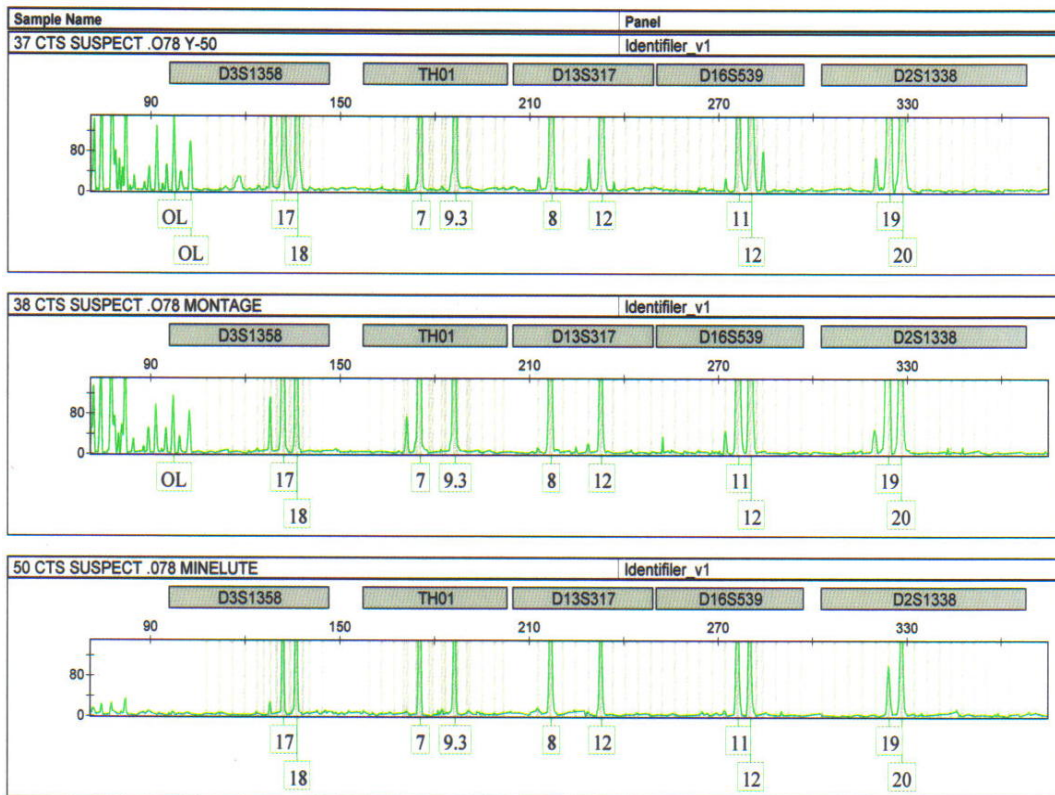
Figure 11: VIC dye artifacts observed at D3S1358. Top row Microcon-50, center row Montage PCR® and bottom row MinElute purifications.

Efficacy of Post PCR Purification using a Silica Gel Membrane (MinElute)

MinElute-purification of the PCR products yielded full DNA profiles from each amplificatin at 78 pg DNA. At 39 pg partial profiles detecting 90-93% of the alleles were obtained while genetic information from as little as 5-10 pg of input DNA was generated (Table 1). This was accomplished by eluting into 25 µL of EB buffer, and injecting 1.5 µL of the purified eluate with 25 µL of formamide and 0.1 µL LIZ size standard. In contrast, the standard method without PCR purification yielded full, albeit weak, profiles with 156 pg of DNA and partial profiles with 39 pg of DNA (Table 1). Allelic signal increases from 3.4 to 4.9 fold (mean = 3.9) were obtained by PCR purification compared to unpurified product (Table 2). This increase in fluorescent signal intensity produced off scale data 625 pg DNA. There did not appear to be a relationship between the fold increase in signal intensity and the input quantity of DNA. A significant reduction in primer peak product was observed after post PCR purification compared to not having a purification step (Figure 12).

Figure 12: Comparison of purified and unpurified primer peaks. Raw date from injecting 1.5 µL of unpurified product on the left (A). Raw data from injecting 1.5 µL of un-concentrated (25 µL eluate) MinElute purified product on the right (B).

Use of Concentrated. Purified, Total PCR Product

The effects of injecting the entire MinElute purified product were investigated. MinElute purified DNA (5-78 pg) was eluted into 10 µL of EB buffer,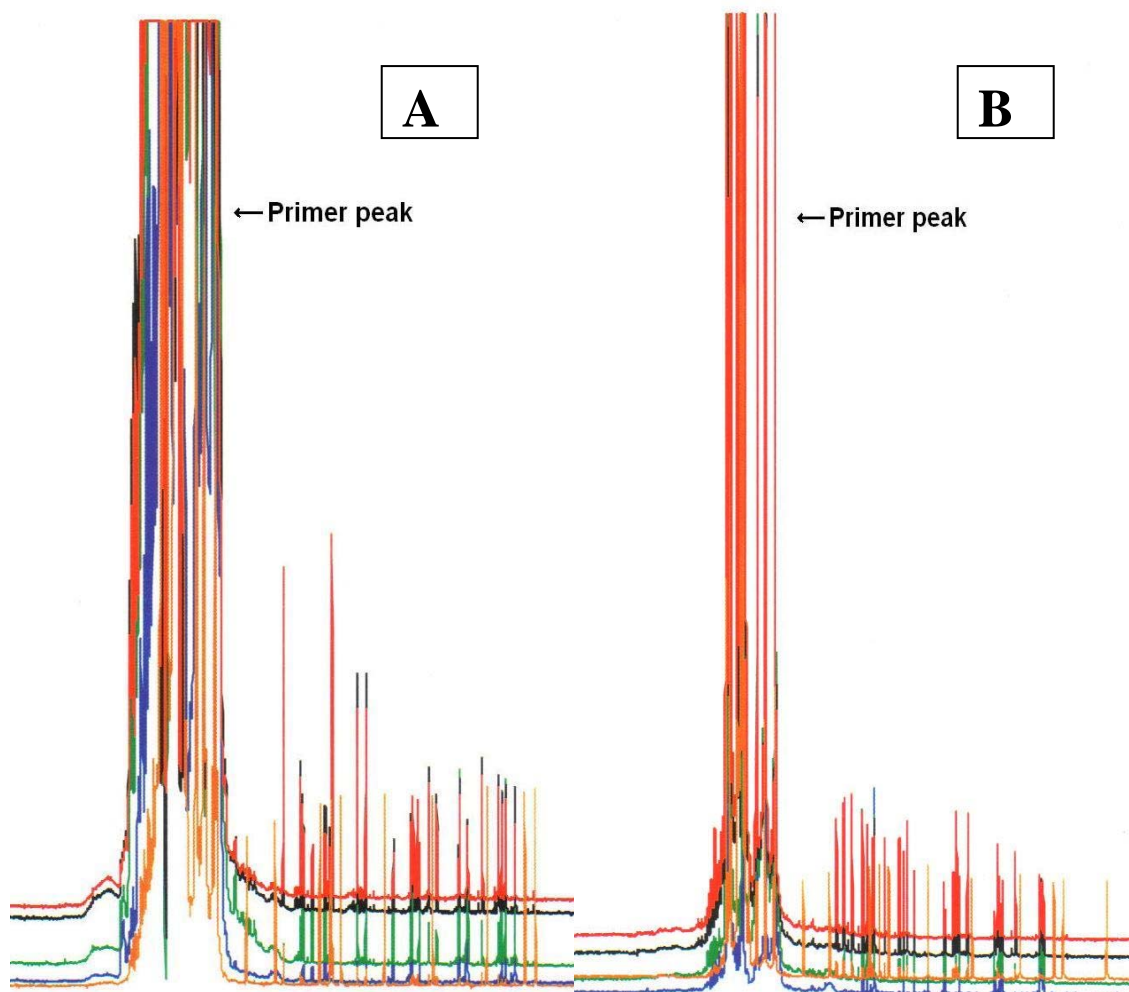 and all of it (i.e. 10 µL) was added to 15 µL of formamide with 0.2 µL LIZ size standard and injected. For comparison, 5-625 pg DNA was amplified in duplicate and, without post-PCR clean up, concentrated to 10 µL in a heat assisted rotary evaporator. The 10 µL of resulting unpurified PCR product were added to 15 µL of formamide and 1.5 µL LIZ size standard and injected.

Significantly, complete DNA profiles were obtained from all amplifications at 20 pg of template DNA when injecting the entire PCR purified product (Figures 13 and 14). This is the equivalent of consistent full profiles from 3-4 diploid cells without the use of increased amplification cycles. Partial profiles were obtained at 10 pg and 5 pg DNA. At 10 pg 73-90% of the alleles were detected and at 5 pg DNA 40-90% of the alleles were detected (Table 1). Allelic signal increases from 16.9 to 21.7 fold (mean = 18.7) were obtained by this process of PCR purification and injecting the total concentrated purified product, compared to standard methods injecting 1.5µL of amplified product without PCR purification (Table 2). Fluorescent allelic signal intensity was increased such that off scale data was produced with 78 pg of DNA when the entire purified product was added.
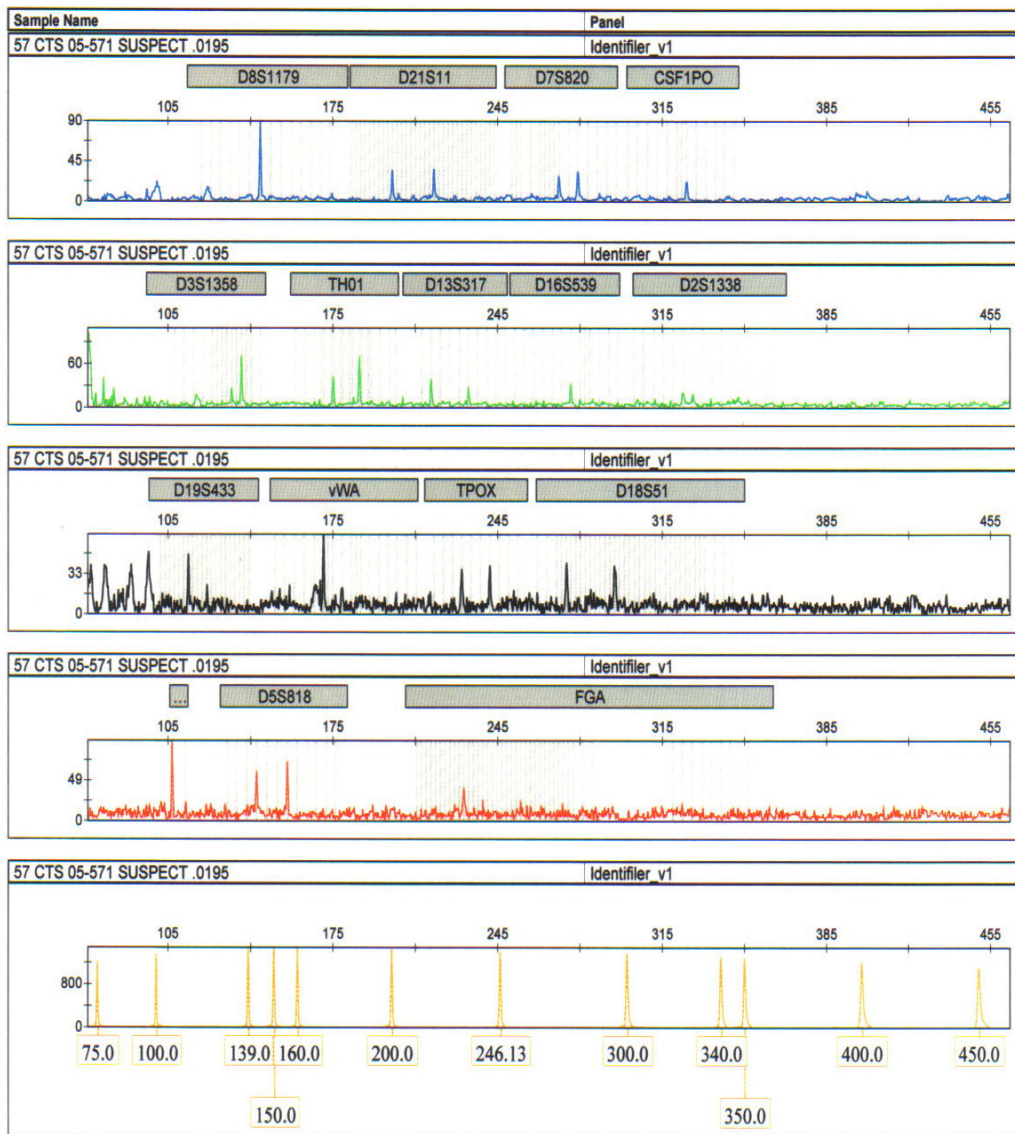
Figure 13: STR profile from 20 pg DNA without post PCR purification
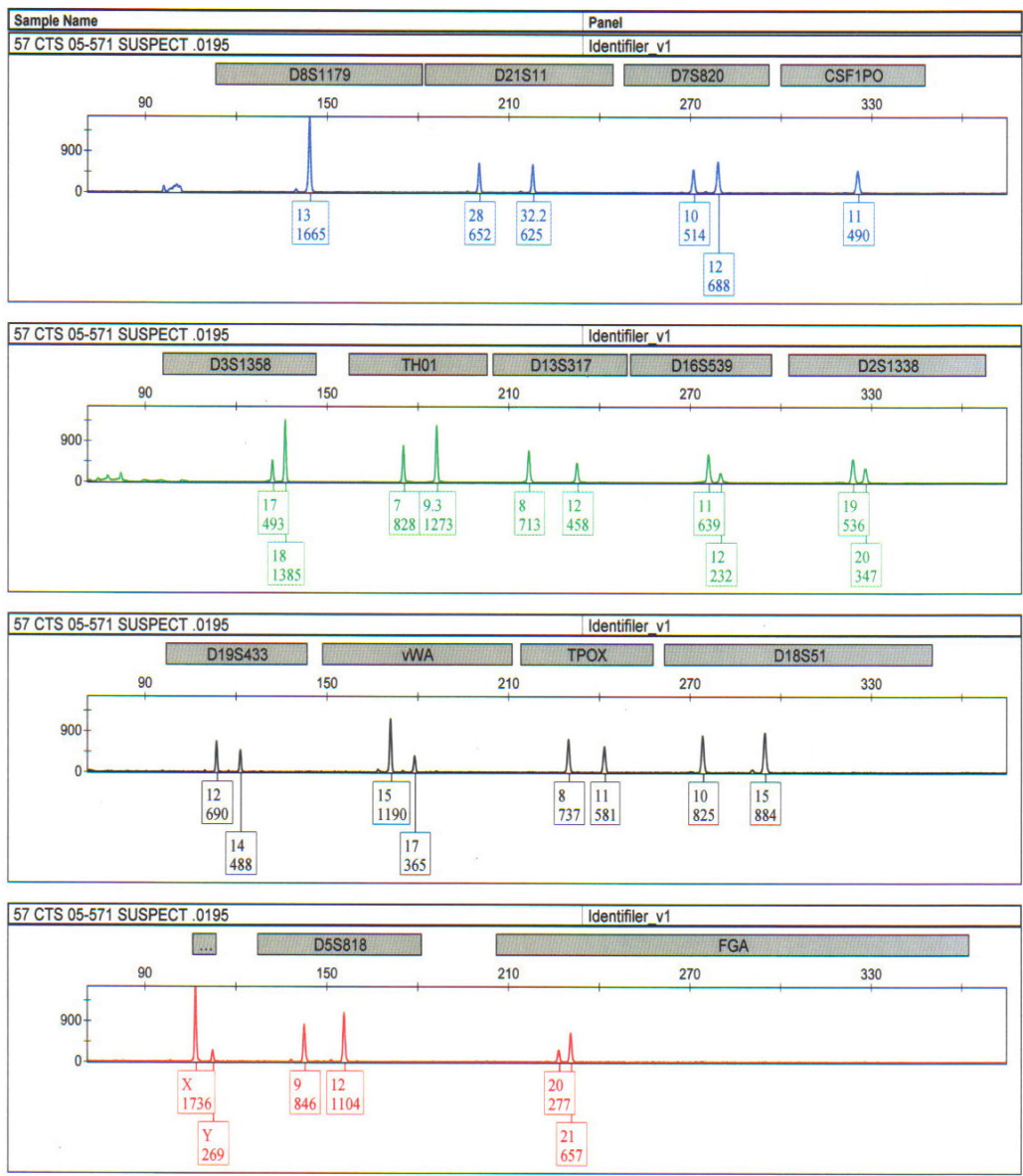
Figure 14: STR profile from 20 pg DNA (same sample as in figure 3) after post PCR purification using MinElute and injection of the entire concentrated purified product.

Again there did not appear to be a relationship between the amount of signal increase and the input quantity of DNA. Direct comparison with injection of the total, unpurified amplification product was not possible since such treatment retarded migration of the alleles approximately 1.5-2 bp such that sample alleles did not align with the allelic ladder bins. In addition, RFUs were decreased relative to the normal injection of 1.5 µL and spurious peaks were created.

Table 1: Increased sensitivity with post PCR purification. Number of alleles detected out of 30 possible alleles. Each concentration represents 4 amplifications (2 extracts amplified in duplicate). Complete profiles were obtained for all samples amplified with 625-312 pg.

PCR Product	156 pg	78 pg	39 pg	20 pg	10 pg	5 pg
1.5uL Unpurified	30	15-25	5-9	0-1	0	0
1.5uL Purified	30	30	27-28	9-19	5-13	0-5
Entire Purified Product	N/D	30	30	30	22-28	12-27

Table 2: Fold increase in fluorescent signal intensity injecting 1.5 µL of purified product and the entire concentrated purified product.

PCR Product	625pg	312pg	156pg	78pg	39pg	20pg	10pg
1.5µL purified	4.9	3.5	3.4	3.8	3.7	4.2	4.3
Entire Purified Product	N/D	N/D	17.2	21.7	18.9	16.9	N/D

Injecting the entire purified product concentrates the residual primers such that the primer peak resembles the primer peak seen with the injection of 1.5 µL unpurified product (Figure 15). Therefore methodologies developed for more complete primer removal would be expected increase the sensitivity of this technique.
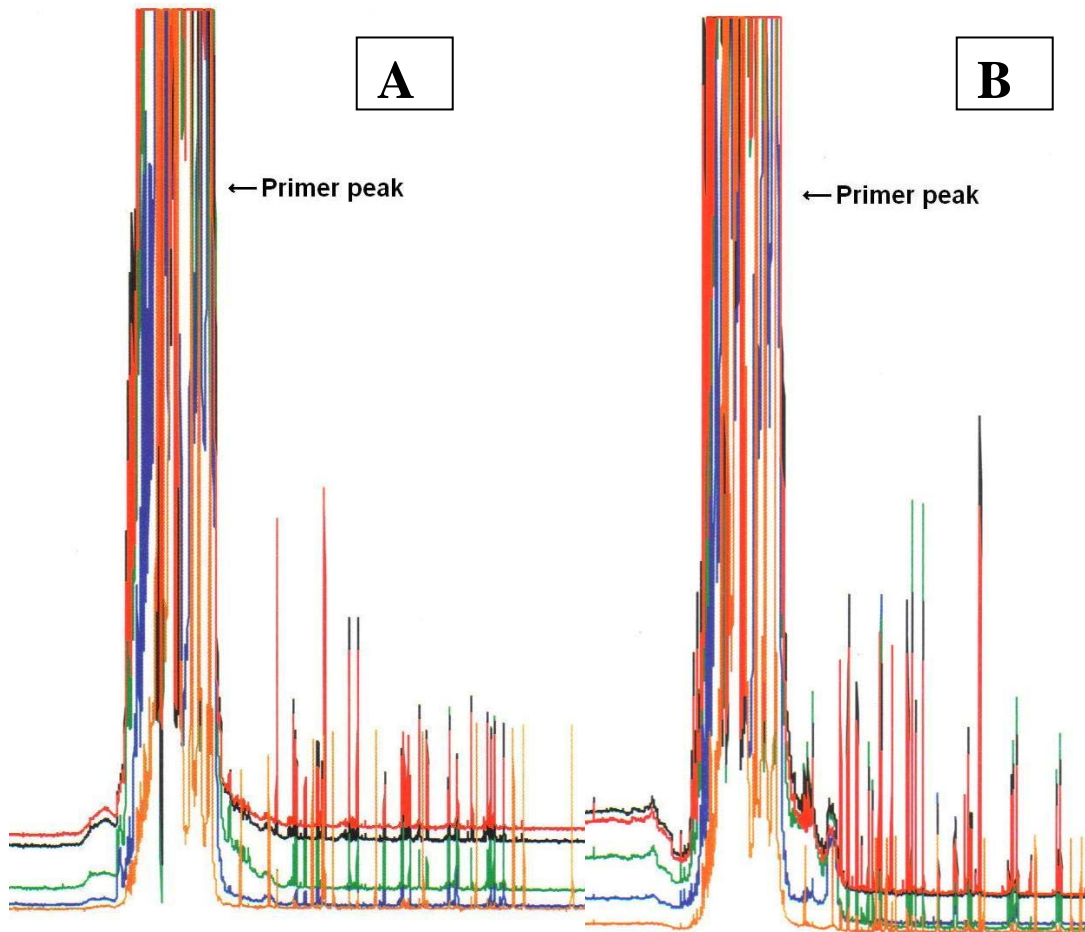
Figure 15: Raw data of primer peaks comparing the injection of unpurified and total concentrated purified PCR product. 1.5 µL unpurified product on the left (A). Entire concentrated purified product on the right (B).

Stochastic LCN-Like Artifacts

Stutter

Increased stutter was observed in approximately 25% of the samples (6/24) when injecting the entire concentrated purified product, representing 0.8% of the allele calls (6/720). Stutter was observed when injecting 1.5 µL of the purified product eluted into 25 µL at the locus D5S818 in only 0.1% of the allele calls (1/900). The increased stutter observed was often seen on re-injection, but not when re-amplified indicating the stochastic nature of the process.

Stutter is a by-product of the polymerase chain reaction. Slipped-strand mispairing is the mechanism believed to be responsible for stutter formation. During the extension cycle the polymerase may fall off allowing the template and extension product to breathe apart. When re-annealing occurs one repeat unit forms a loop. If this loop occurs on the template strand it will result in an extension product one repeat unit shorter than the true allele (n-1). This is the most common stutter product. Conversely, if the loop occurs on the extension strand the product will be one repeat unit longer than the true allele (n+1) (Figure 16).
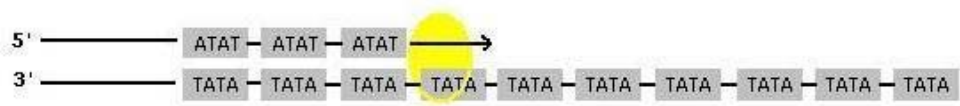
Normal Replication
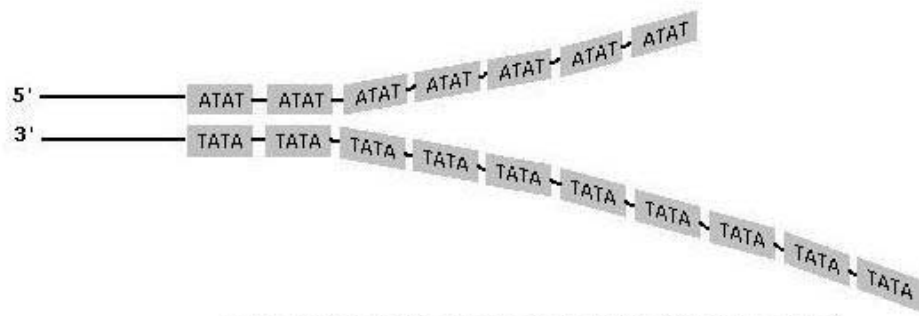
Unpairing of template and extension procduct
that allows for slipped-strand mispairing
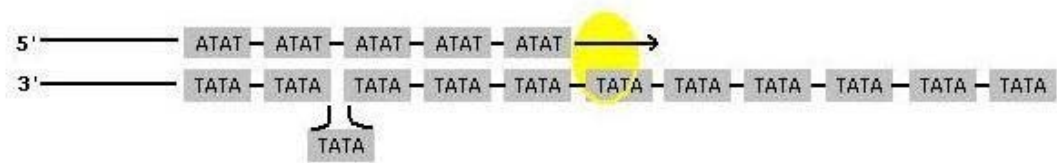
Figure 16: Formation of n-1 and n+1 stutter products.

The stutter products continue to be amplified along with the true allele in the remaining cycles. Normally stutter for tetranucleotide repeats does not exceed 15% of the true allele. However, with LCN analysis amplification template may only be 1 - 4 genomic copies of DNA (5 – 25 pg DNA). When stutter occurs it represents a much larger percentage of the down stream template therefore higher stutter percentages are likely to occur. This is particularly true if slippage occurs early or often in the amplification process. Indeed Whitaker (20) acknowledges the theoretical event of a stutter peak in the absence of the true allele.

44

A more comprehensive stutter study was undertaken to compare stutter values between purified and unpurified product. Fifty known samples of unpurified and fifty known samples of concentrated purified PCR product (10µL of eluate injecting 4-10µL) were examined. At each locus the mean stutter percentage was similar between purified and non-purified PCR product, although PCR purification produced an increase in the variation of stutter at each locus (Figure 17).
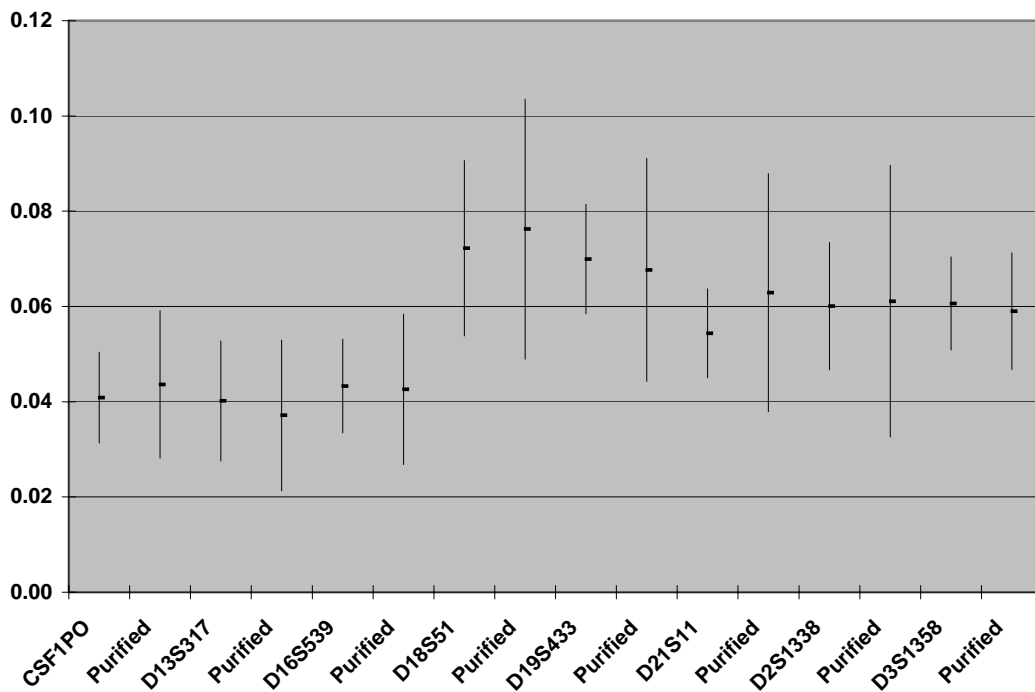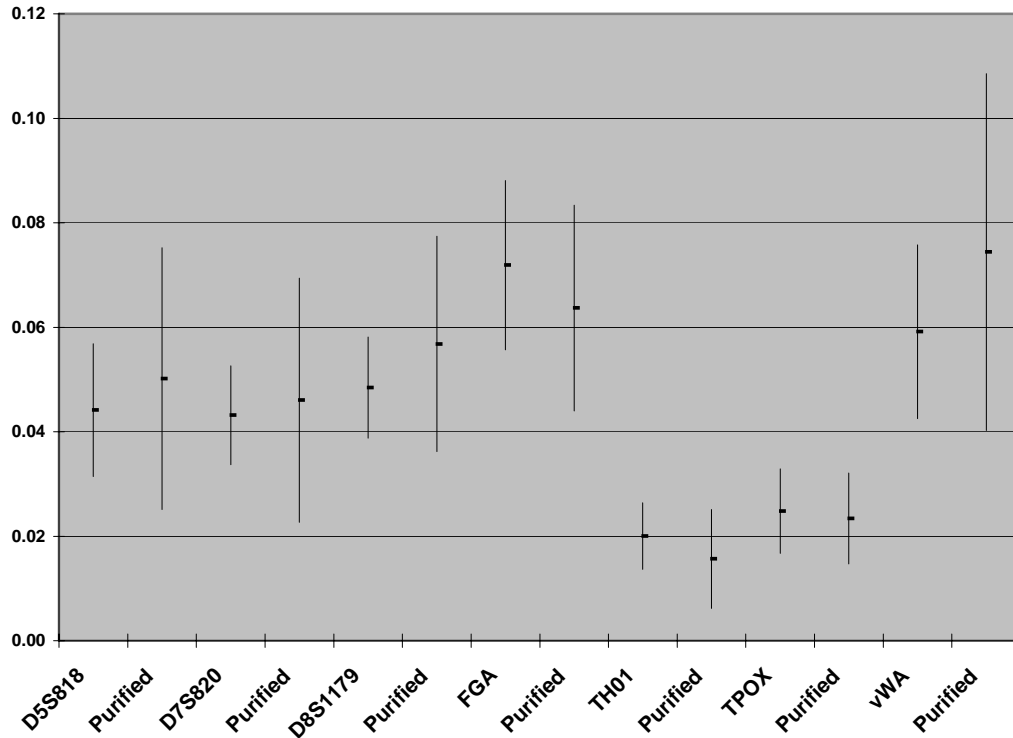
Figure 17: Comparison of stutter before and after purification for each locus. The average stutter percentage is indicated, with the length of the bar representing one standard deviation either side of the mean.

In this study, 3.6% of the stutter values (37/1026) for the purified product exceeded Identifiler cut off values at the loci D5S818 (9), D21S11 (8), D8S1179 (5), D19S433 (4), D2S1388 (3), vWA (3), D7S820 (2), CSF1PO (1), TH01 (1), and TPOX (1). The unpurified product exceeded Identifiler cut of values 0.4% of the

46

time (4/933) at the loci D5S818 (2) and D8S1179 (2). The highest stutter recorded for the purified product was 29.7% at vWA. For A detailed view of purified PCR product stutter results by locus see the Appendix.

Stutter peaks are normally in the n-1 position, where n = the allele (in repeat units). When injecting concentrated purified product (10 µL eluate injecting 1.5-10 µL) an n+1 stutter peak was observed in approximately 7% of the samples (7/105). However these n+1 peaks, although replicated upon re-injection, not appear in duplicate amplifications. When stutter ratios exceed software filter values or n+1 stutter is present, concentration of the purified product can elevate stutter peaks above threshold (100 RFU). Therefore the vast majority of the increased stutter observed (including the formation of n+1 peaks) arose from the use of 4-10 µL of concentrated purified product.

Allele Drop-in

In three of the sixty amplifications injecting 4-10µL of concentrated purified product an unexpected allele was obtained at the locus D2S1338 (Figure 18). No additional peaks below threshold were observed in these amplifications and the negative amplification and reagent blank controls were clean. The drop-in alleles were not reproducible upon re-amplification.
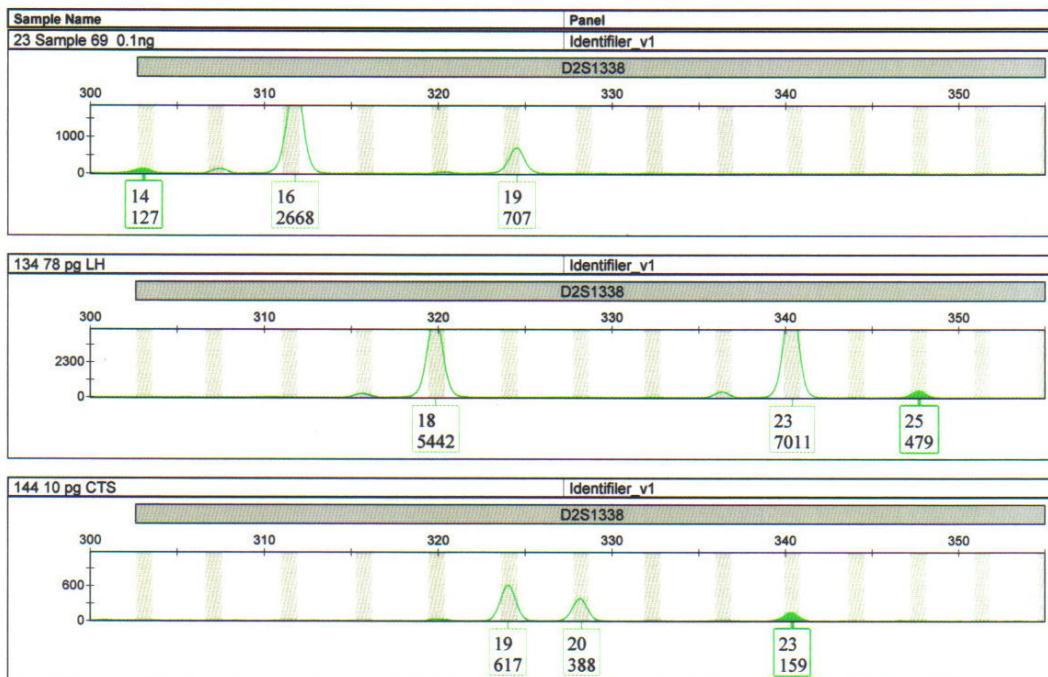
Figure 18: Allele drop-in observed at the D2S1338 locus. Drop-in alleles 14, 25 and 23 are highlighted in each panel with peak heights indicated beneath the allele call.

Heterozygote Peak Height Imbalance

Heterozygote peak imbalance in low template samples can be extreme (7, 12, 20). Random PCR amplification of one allele during the early cycles of PCR can cause preferential amplification of that allele over the sister allele at heterozygous loci. This effect is expected to become more pronounced as template copy decreases. Heterozygote peak imbalance in its extreme form results in allelic drop-out. A comparison of heterozygous peak imbalance was made between unpurified product and purified product. The peak height ratio (PHR) of

heterozygous loci was calculated by dividing the peak height of the lowest allele by the peak height of the highest allele (PHR= PH_{low}/PH_{high}) such that the PHR is always less than or equal to 1, with 1 representing perfect peak height balance. Heterozygous loci exhibiting allelic drop-out at 10 pg and 5 pg were not included in the calculations. Peak height ratios for purified product were obtained by injecting the entire purified product using input quantities of 78 pg (n=56), 39 pg (n=56), 20 pg (n=56), 10 pg (n=39), and 5 pg (n=25). Four amplifications for each input quantity of DNA were analyzed. Peak height ratios for unpurified product were obtained from 50 amplifications using 1 ng DNA (n=564). Not surprisingly, the average peak height balance decreased from 88% for unpurified samples to 52% for purified samples with input DNA of 5 pg (Figure 19).

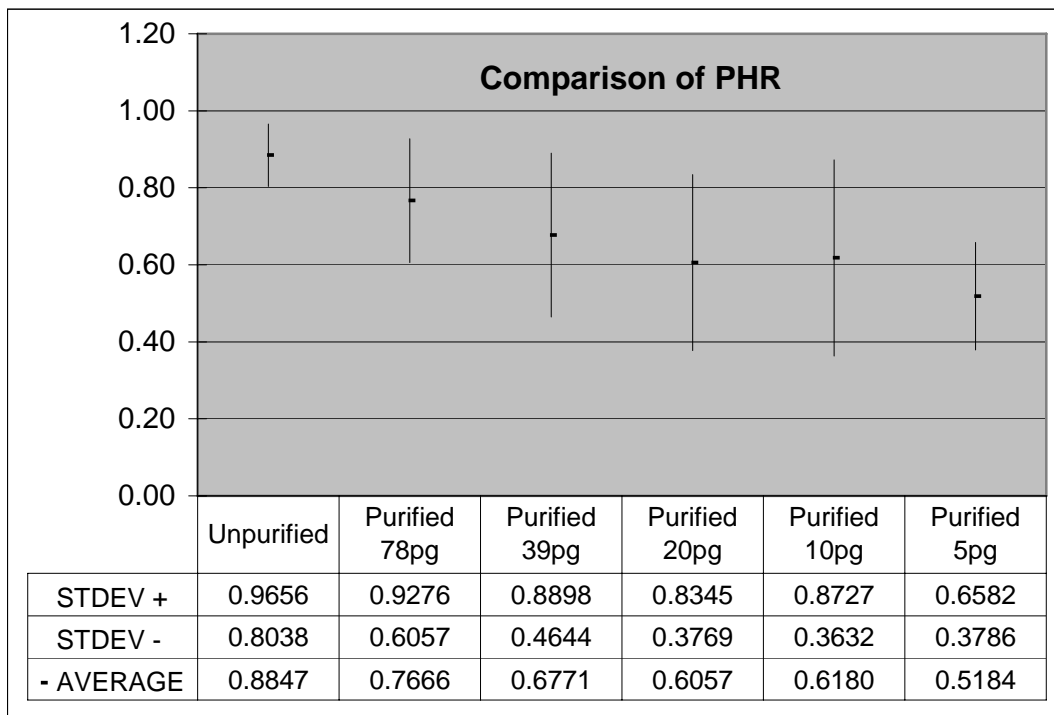|	Unpurified	Purified 78pg	Purified 39pg	Purified 20pg	Purified 10pg	Purified 5pg
STDEV +	0.9656	0.9276	0.8898	0.8345	0.8727	0.6582
STDEV -	0.8038	0.6057	0.4644	0.3769	0.3632	0.3786
- AVERAGE	0.8847	0.7666	0.6771	0.6057	0.6180	0.5184

Chart title: **Comparison of PHR**

Figure 19: Comparison of STR heterozygote loci peak imbalance with purified and non-purified PCR products. Unpurified n = 564. Purified 78 pg, 39 pg, and 20 pg n = 56; 10 pg n = 39; 5 pg n = 25.

Heterozygote peak imbalance ranged from 58-100% for unpurified samples to 15-99% for purified product amplified with 10 pg (Table 3).

Table 3: Range of heterozygote imbalance observed between amplifications of 1 ng unpurified PCR product and 78 pg, 39 pg, 20 pg, 10 pg, and 5 pg of purified PCR product.

PCR Product	Unpurified 1ng	Purified 78pg	Purified 39pg	Purified 20pg	Purified 10pg	Purified 5pg
PHR Range	58-100%	34-99%	22-99%	16-97%	15-99%	31-80%

Non-Probative Case Type Samples

To assess the effectiveness of PCR purification on casework type samples two dermal ridge fingerprints (on paper and glass substrates) and a telogen hair root were collected from three subjects. A comparison of the profiles obtained from the dermal ridge fingerprint samples before and after PCR purification showed that significant allelic data was obtained from the purified product when little or no allelic data was obtained prior to PCR purification (Table 4). Figures 20 and 21 demonstrate electropherograms obtained from a fingerprint swabbed from a glass slide before and after post PCR purification. The entire concentrated purified product was injected for these samples. The samples were amplified in duplicate.

Results for each amplification of the unpurified product are reported while only

duplicated alleles are reported for the purified samples.

Table 4: Comparison of unpurified and entire concentrated purified PCR product in non-probative case type samples. The results of each amplification are separated by a / in the unpurified product columns. The purified product columns only reflect the duplicated alleles.

SUBJECT 1	Subject 1 Profile	Fingerprint paper Unpurified	Fingerprint paper Purified	Fingerprint glass Unpurified	Fingerprint glass Purified
D8S1170	12, 14	* / *	12, 14	* / *	12, 14
D21S11	30, 31	* / *	30, 31	* / *	30
D7S820	8, 11	* / *	11	* / *	*
CSF1PO	10, 12	* / *	10, 12	* / *	*
D3S1358	16, 16	16 / 16	16	16 / 16	16
TH01	6, 8	8 / *	6, 8	* / *	*
D13S317	11, 11	11 / *	11	* / *	11
D16S539	9, 13	* / *	9, 13	* / *	9
D2S1338	25, 25	* / *	25	* / *	*
D19S433	15, 15	15 / 15	15	* / *	15
vWA	15, 17	* / 15	15, 17	* / *	15, 17
TPOX	8, 11	* / *	8, 11	* / *	11
D18S51	12, 12	* / *	12	* / *	*
AMEL	X Y	X / X	X Y	* / *	X Y
D5S818	11, 12	12,/ 11	11, 12	* / *	11, 12
FGA	21, 24	* / *	21, 24	* / *	24

The asterisk (*) represents no data obtained.

SUBJECT 2	Subject 2 Profile	Fingerprint paper Unpurified	Fingerprint paper Purified	Fingerprint glass Unpurified	Fingerprint glass Purified
D8S1170	13, 15	* / *	13, 15	* / *	13, 15
D21S11	29, 31.2	* / *	31.2	* / *	29, 31.2
D7S820	11, 11	* / *	*	* / *	11
CSF1PO	11, 11	* / *	11	* / *	11
D3S1358	14, 16	14 / *	14, 16	* / *	14, 16
TH01	7, 9.3	* / *	7, 9.3	* / *	7, 9.3
D13S317	9, 11	* / *	11	* / *	9, 11
D16S539	13, 13	* / *	13	* / *	13
D2S1338	17, 20	* / *	20	* / *	17, 20
D19S433	12, 13	13 / *	12, 13	* / *	12, 13
vWA	16, 18	* / *	16, 18	* / *	16, 18
TPOX	8, 11	* / *	8	* / *	8
D18S51	12, 13	* / *	12, 13	* / *	12, 13
AMEL	X Y	* / *	Y	* / *	X
D5S818	12, 12	* / 12	12	* / *	11, 12
FGA	20, 23	* / *	20, 23	* / *	20

| SUBJECT 3	Subject 3 Profile	Fingerprint paper Unpurified	Fingerprint paper Purified	Fingerprint glass Unpurified	Fingerprint glass Purified
D8S1170	9, 15	* / *	*	* / *	9, 15
D21S11	29, 29	* / *	29	* / *	*
D7S820	8, 10	* / *	10	* / *	8, 10
CSF1PO	12, 13	* / *	*	* / *	12
D3S1358	15, 15	* / *	16	* / *	15
TH01	6, 9	* / *	6, 9, 9.3	* / *	*
D13S317	12, 13	* / *	*	* / *	12, 13
D16S539	11, 12	* / *	11	* / *	11
D2S1338	19, 20	* / *	19	* / *	18, 19
D19S433	12, 16	* / *	*	* / *	16
vWA	17, 18	* / *	17, 18	* / *	18
TPOX	8, 11	* / 8	*	* / 8	8
D18S51	12, 15	* / *	*	* / *	12, 15
AMEL	X X	* / *	*	* / *	X
D5S818	11, 12	* / *	*	12 / *	*
FGA	19, 25	* / *	*	* / *	*
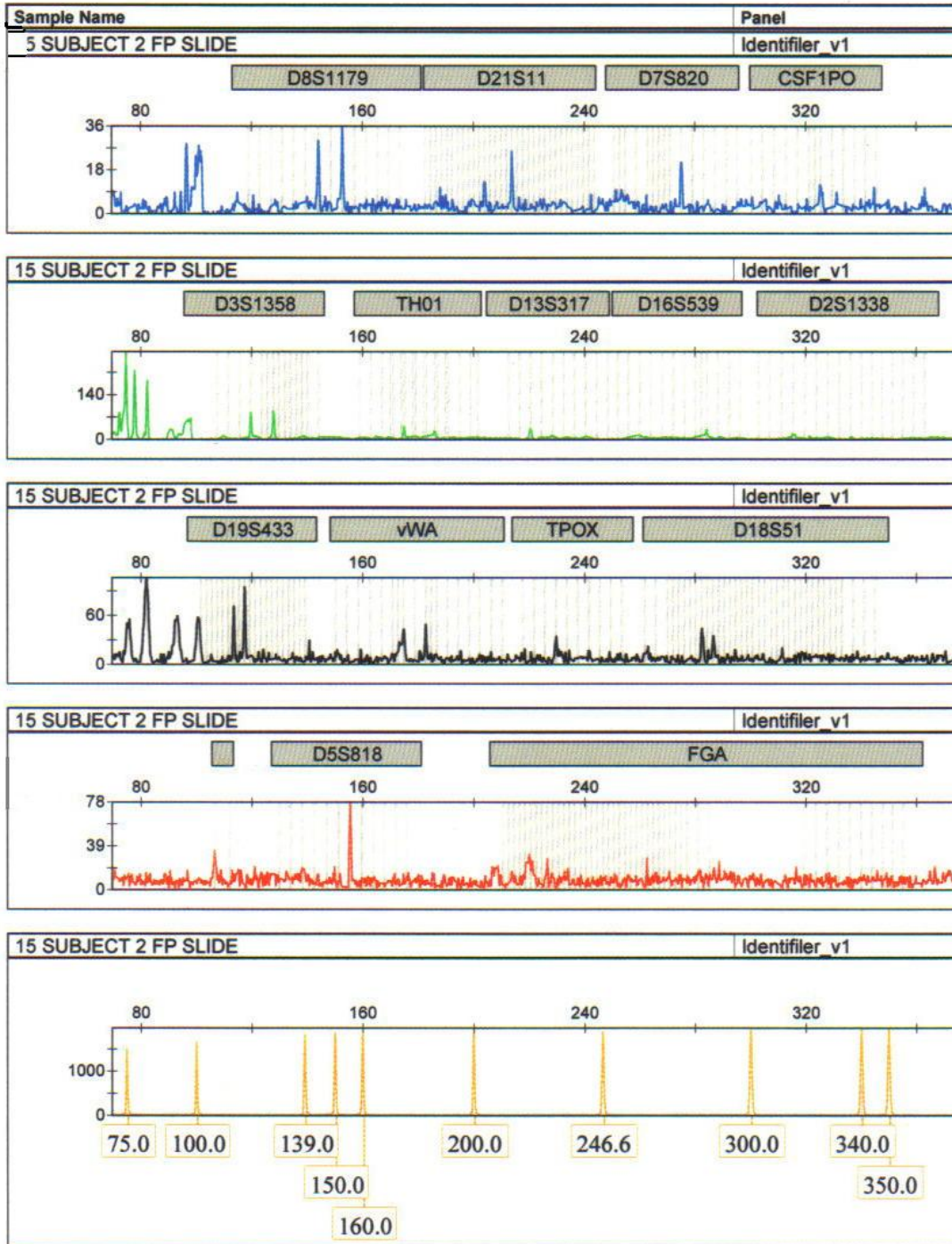
Figure 20: STR profile of fingerprint on a glass slide before PCR purification
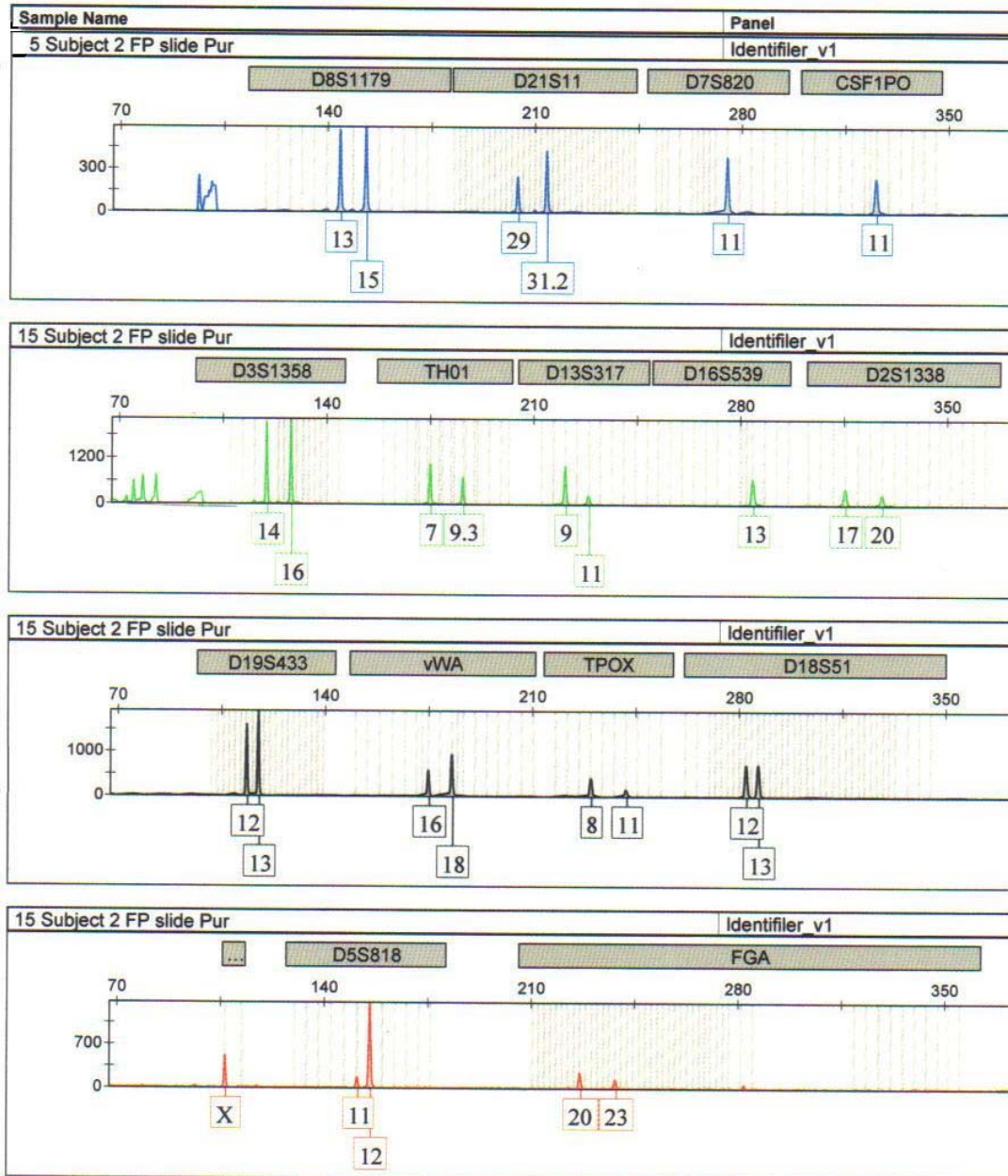
Figure 21: STR profile from fingerprint (same sample as above) after PCR purification and injection of the entire concentrated purified product. Note the drop-out of the Y allele.

The fingerprint on glass samples from subjects 2 and 3 exhibited elevated stutter at D5S818 and D2S1331 that also appeared in the duplicate amplification. In our stutter study of purified samples D5S818 had the highest stutter filter failure rate at 12.7% and was one of the two loci with stutter filter failure in the unpurified samples. D2S1331 exhibited elevated stutter in 4.5% of the purified samples in our study.

The fingerprint on paper sample for subject 3 indicated the presence of a contaminating profile. The 9.3 allele at TH01 and the 16 allele at D3S1358 were the only alleles duplicated. Contamination was reflected in both amplifications and may have arisen from secondary transfer, a contaminant in the substrate or extraction tubes, or laboratory contamination. The contaminating alleles were not concordant with subjects 1 and 2 or with laboratory personnel. Two alleles were detected in a purified fingerprint reagent blank, but did not appear in the duplicate amplification. These alleles were not concordant with the contaminating profile.

No alleles were detected in the telogen root hair samples before purification. A total of 8 alleles, concordant with the known profiles, were detected in the six amplifications after purification. However, no allele was reproduced in the duplicate amplification (data not shown). Electropherograms of unpurified and entire concentrated purified product are shown in Figures 22 and 23.
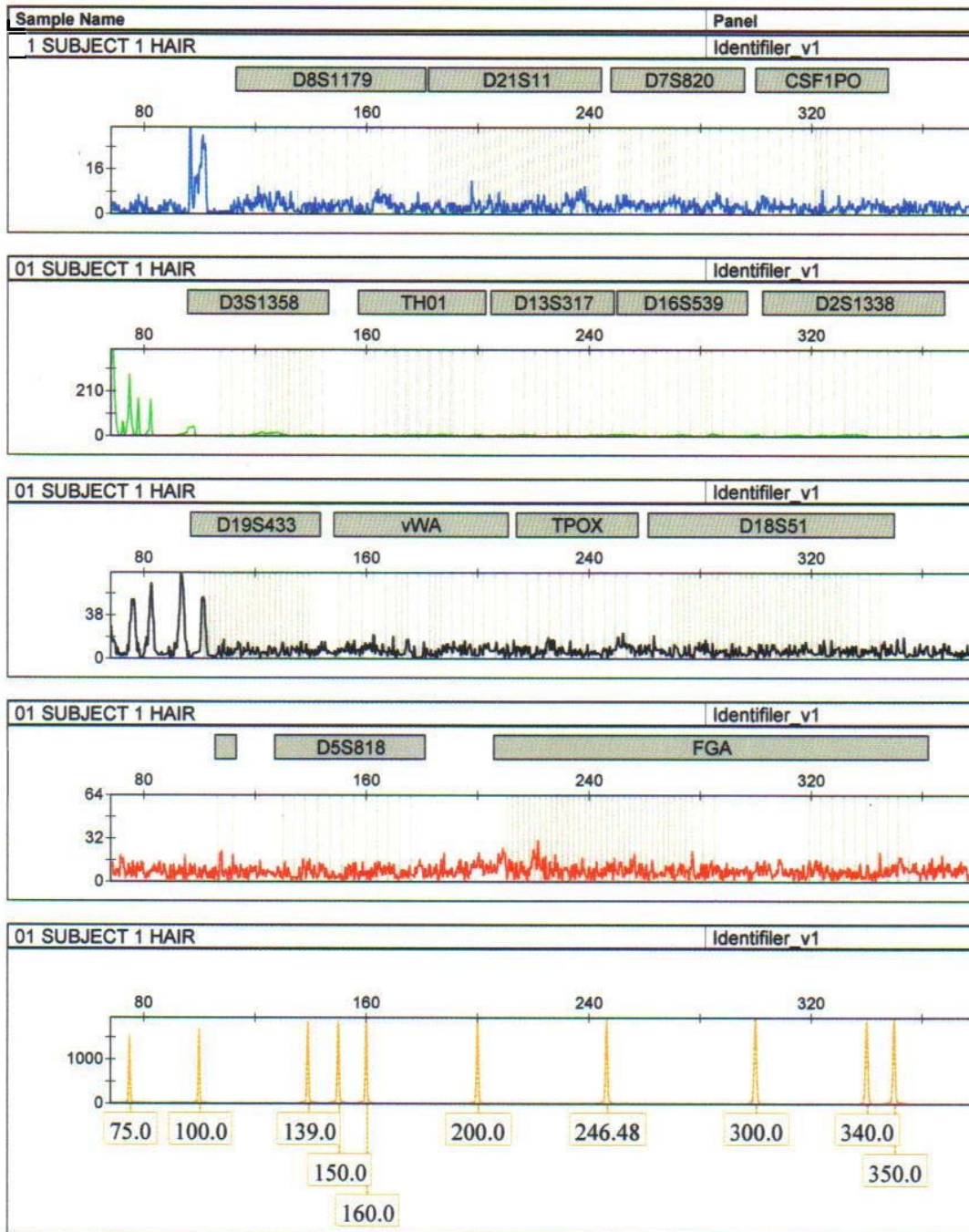
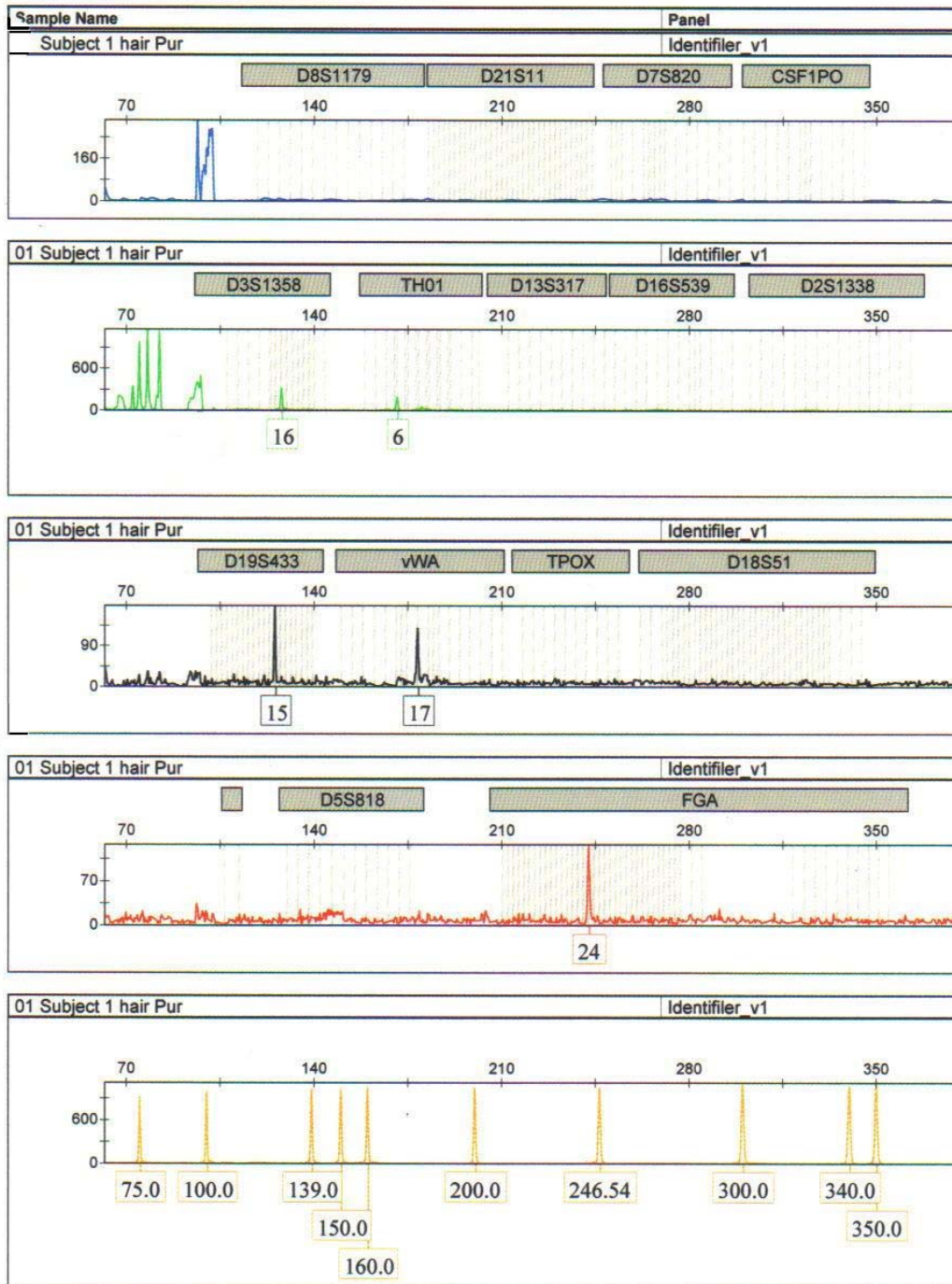Figure 22: Telogen root hair before purification.

Figure 23: Telogen root hair entire concentrated purified PCR product (same sample as above).

CHAPTER FIVE: CONCLUSION

The intent of this study was to evaluate different post PCR purification methods in an attempt to improve the analytical sensitivity of standard STR typing. Four different PCR purification methods (Microcon-50, Montage PCR® filter, MinElute silica column and ExoSAP-IT®) were evaluated. The greatest increase in fluorescent signal intensity was obtained from the Montage PCR® filter. The Montage PCR® filter is suggested over the Microcon-50. However, the presence of non-specific product at low levels in the VIC dye lane at the D3S1358 locus made the Montage PCR® filter unsuitable for studies injecting the entire purified product. ExoSAP-IT® treatment followed by silica gel purification did a superior job removing primer, but the formation of minus A peaks render it unsuitable for fragment length analysis. Based upon the purity of the eluate obtained, effect on signal intensity, and ease of use, the Qiagen MinElute silica column was selected for detailed study. Purified PCR product using this method produced a 4 fold increase in fluorescent signal intensity over unpurified product. Hutchinson (10) subjected microsatellite PCR products to Sephadex™ purification and briefly examined its effect using capillary electrophoresis. A 3.5 fold increase in signal intensity was reported, comparable to our observations of a 4 fold increase using the un-concentrated purified product. Furthermore, by adding the entire concentrated purified PCR product a 19 fold increase in signal intensity can be expected. Using this method complete profiles were obtained from 20 pg DNA (3-4 diploid cells)

and significant allelic data was generated down to 5-10 pg of DNA without the use of increased amplification cycles.

Though great care was taken to observe good laboratory practices, these studies were carried out in a case-working laboratory without specialized precautions to prevent contamination. Incidents of contamination were not observed in the negative amplification controls as reported by Gill et.al. (7, 25) using increased amplification cycles. A single incident of contamination was detected in a reagent blank, but was not duplicated upon re-amplification. This could suggest that there is less risk of detecting adventitious DNA with PCR purification using 28 cycles than with increased cycle amplifications used in conventional LCN analysis. A combination of post PCR purification and increased cycle number may allow for complete profiles from 5 pg templates with greater allele fidelity. The detection of a foreign profile in one of the case type samples implies the greatest risks for contamination occurs in the collection and extraction process as opposed to amplification, purification, and electrophoresis set up. Thus the need for strong contamination prevention guidelines is warranted.

Increased stutter and heterozygote peak imbalance were observed with the use of concentrated purified PCR product on <100 pg DNA. Our stutter study agreed with the observation of Whitaker (20) that stutter means did not vary significantly but stutter variance was increased. The increased incidences of stutter, heterozygote peak imbalance and allelic drop-in are in accordance with the observations of Gill et. al. (6, 7, 12, 20) and support the necessity for having

duplicate amplifications and appropriate interpretation guidelines for LCN-generated profiles.

Guidelines for post PCR purification using the MinElute column are suggested below. Since amplification efficiency can vary from sample to sample, guidelines for post PCR purification are based upon RFU observed rather than input quantities of DNA. Laboratories should establish at what point LCN procedures and interpretation guidelines should be applied and perform validation studies before implementation on casework.

After standard 28 cycle PCR amplification, samples should be injected prior to post PCR purification. In order to minimize the risk of off scale data and maximize allelic information obtained, electropherograms should be examined to determine the highest above threshold and lowest below threshold peak heights. A purification strategy based upon peak heights (RFU) observed can be selected from Table 5.

Table 5: Strategy for post PCR purification of samples.

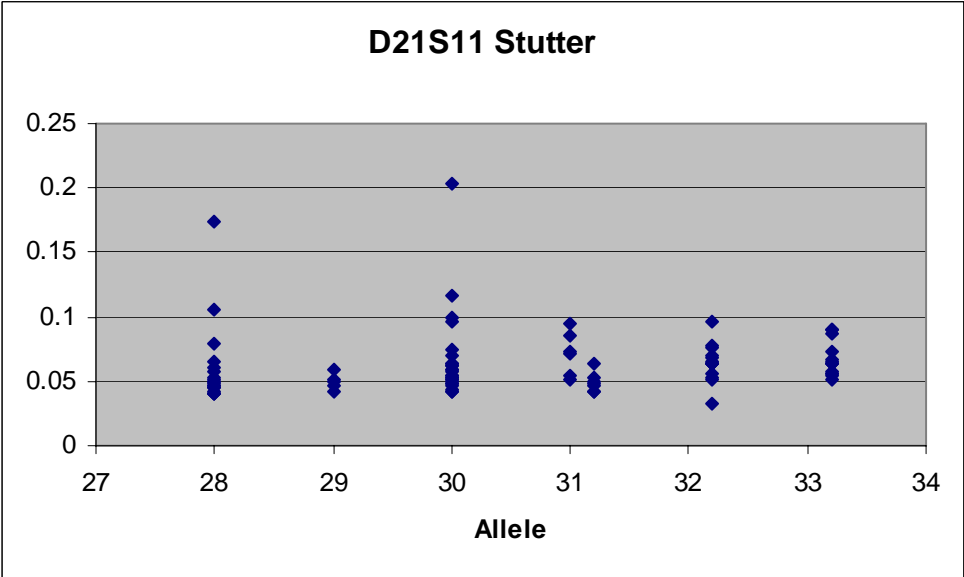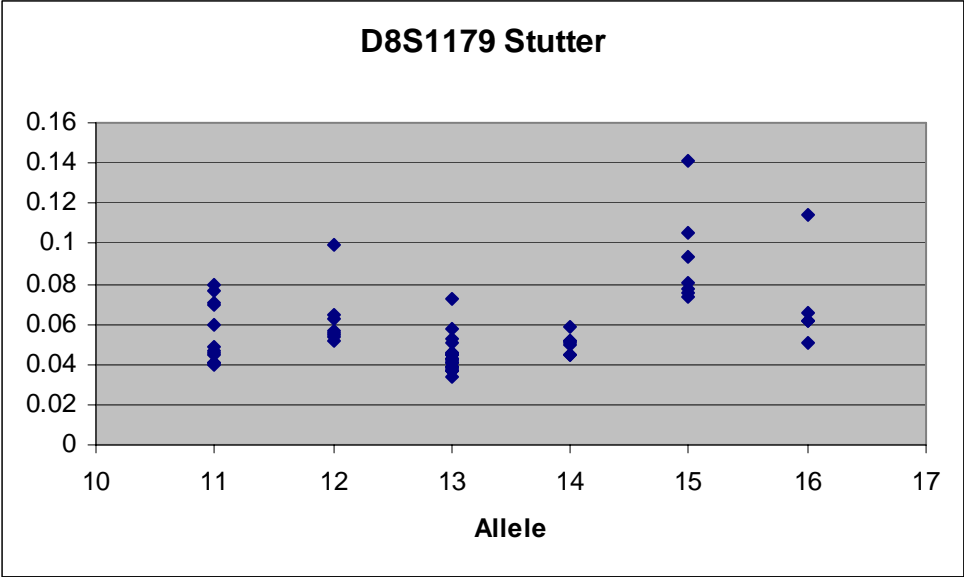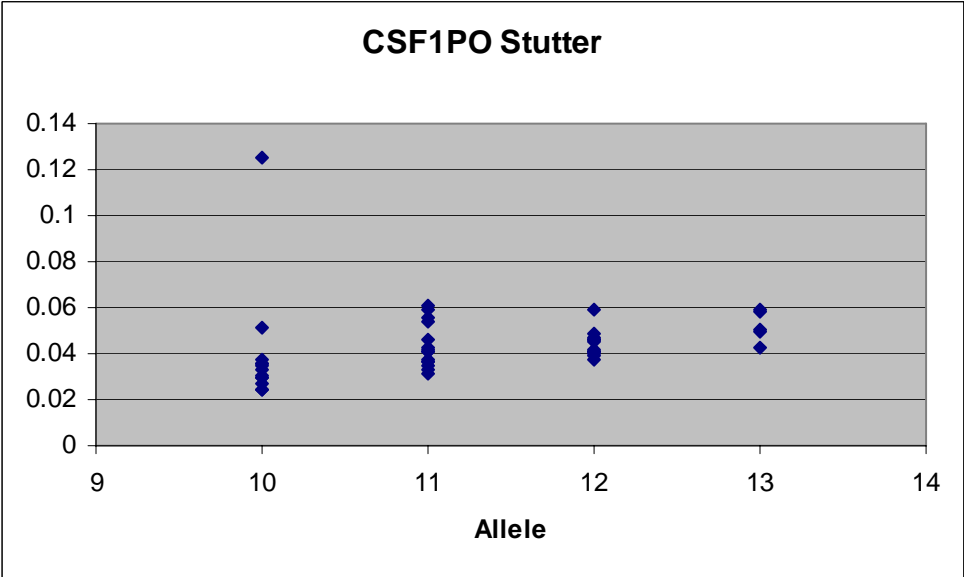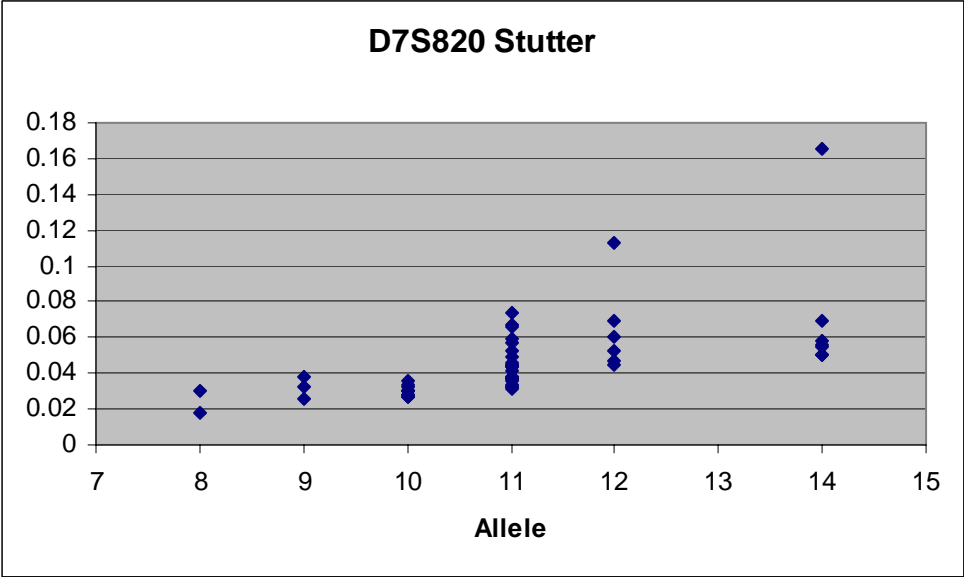| Pre-Purification Peak Heights	$\geq$ 50 RFU and $\leq$ 1,000 RFU	< 600 RFU	< 300 RFU
Volume of Eluate	25 µL	10 µL	10 µL
Volume of Formamide	25 µL	15 µL	15 µL
Volume of Size Standard	0.1 µL	0.1 µL	0.2 µL
Volume of Purified PCR Product	1.5 µL	1.5 µL	10 µL
Average Increase in Fluorescent Signal	4 fold range (3-5)	* 6.5 fold range (5-8)	19 fold range (17-22)

*Data not normalized

This table represents an estimate for results. The efficiency of the purification can vary from sample to sample and the fold increase in fluorescent signal increase can vary across alleles in a profile. If needed fluorescent signal intensity can be optimized by increasing or decreasing the amount of purified product in the formamide mix. Up to a 50:50 mix of purified product and formamide have been injected in this study with good quality results. In addition injection times may be altered to optimize results.
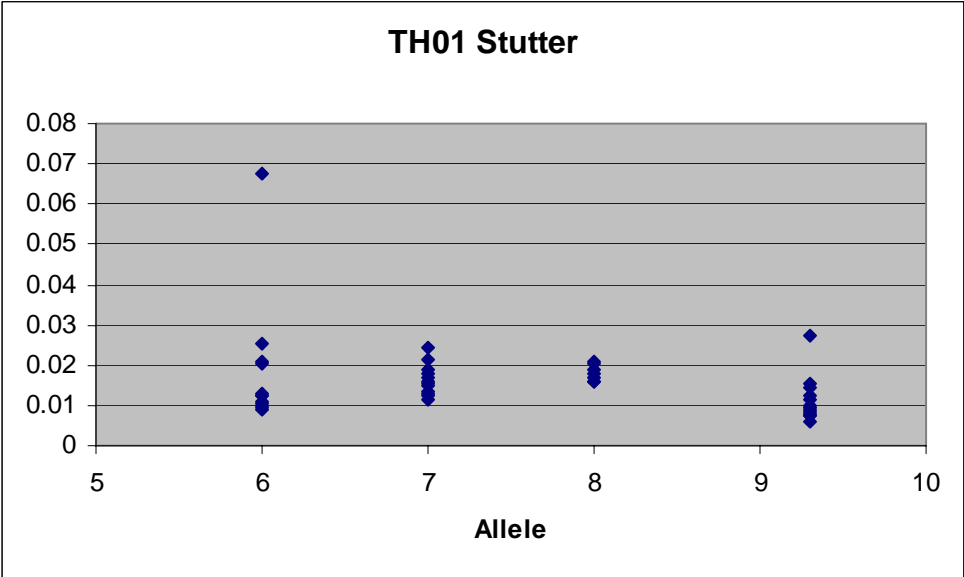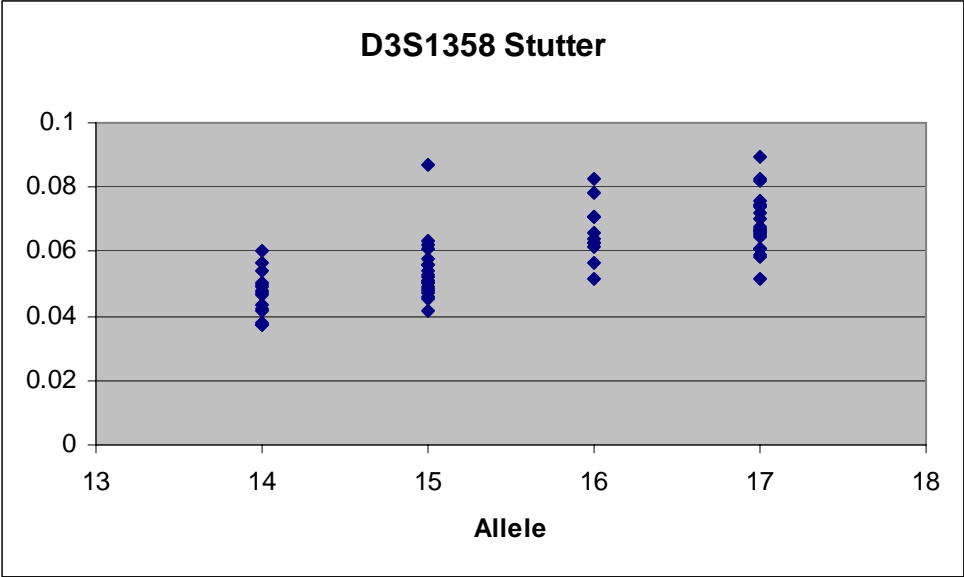
Post PCR purification with the MinElute column can greatly enhance the sensitivity of the PCR process obtaining full profiles down to the 20 pg range and
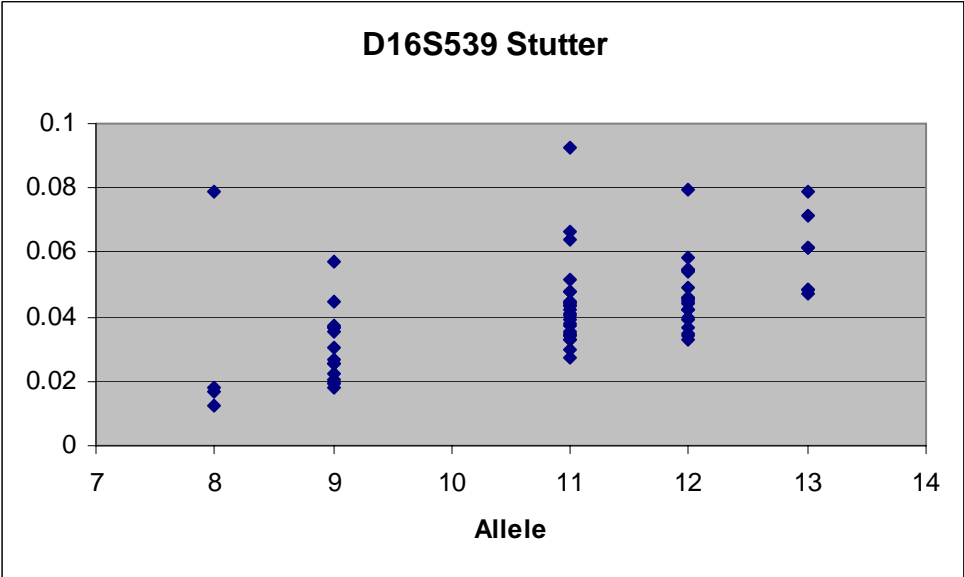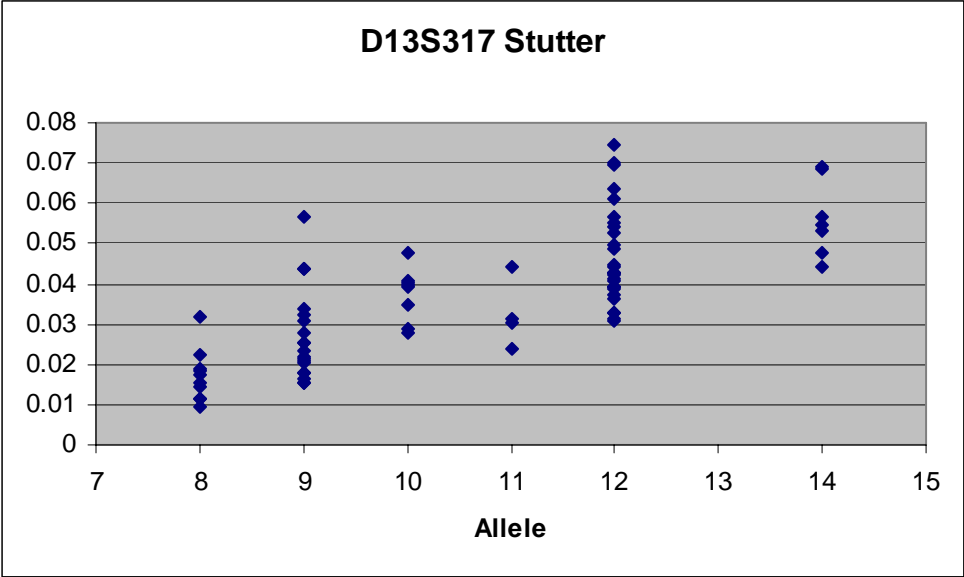
generating significant data down to 5 pg without increasing amplification cycles. This purification method is simple, inexpensive and can be accomplished in about 15 minutes. By adjusting the volume of eluate and the amount of purified product injected, the sensitivity of this technique can be controlled. Thus post PCR purification fits easily into the flow of casework and can be used in a two-fold application, to boost below threshold peaks of weak samples or minor contributors to mixtures and as a technique for LCN analysis.
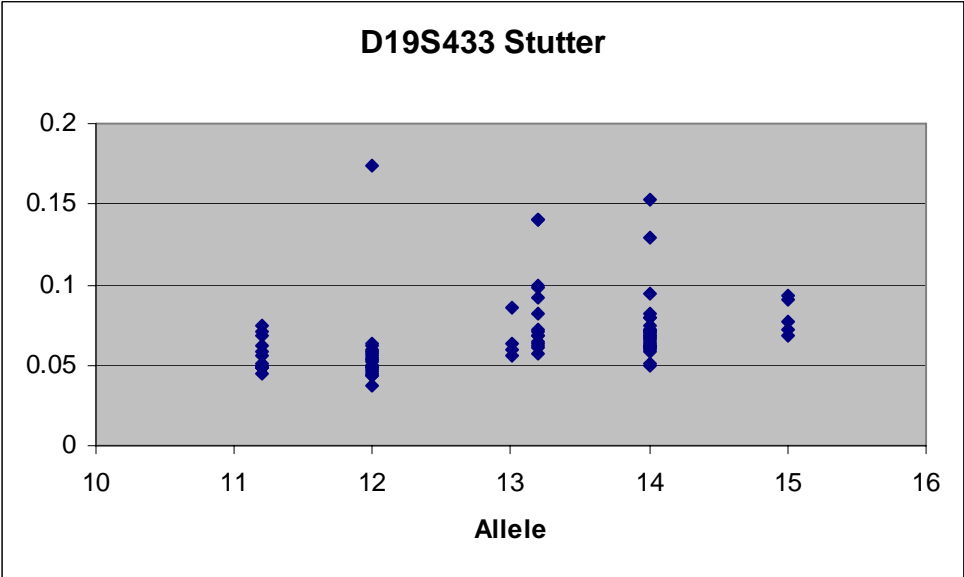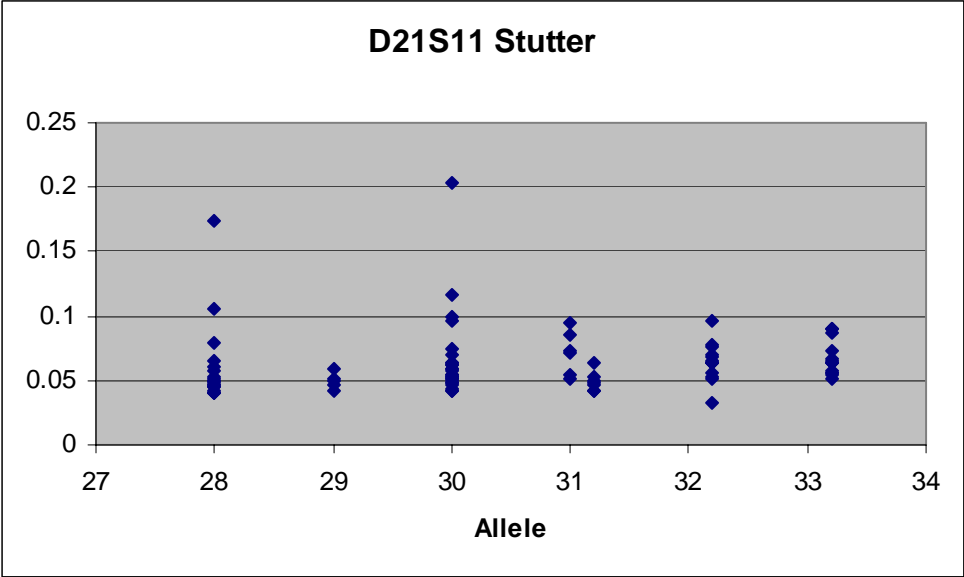
APPENDIX: PURIFIED STUTTER RESULTS BY LOCUS

D8S1179 Stutter

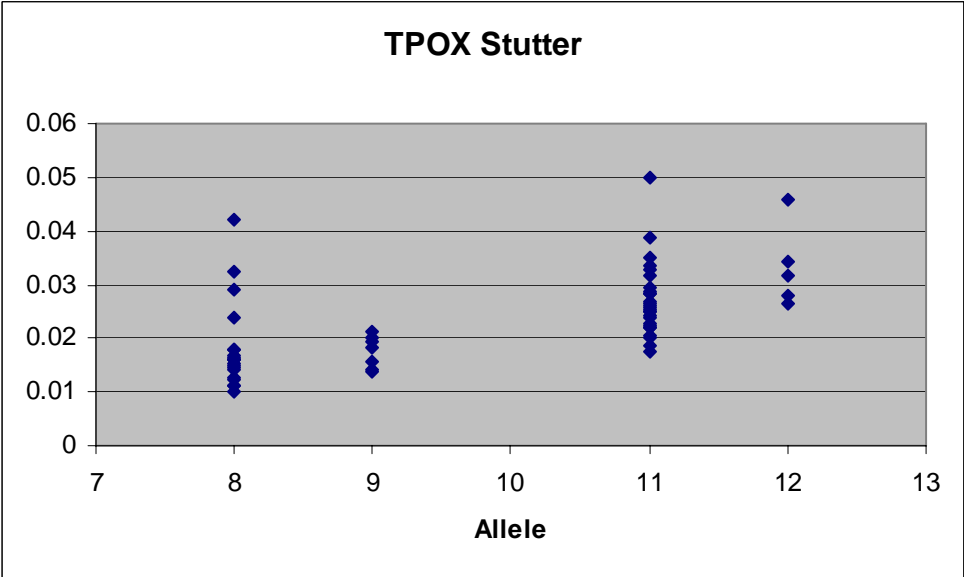**D21S11 Stutter**

D7S820 Stutter

CSF1PO Stutter

D3S1358 Stutter

TH01 Stutter

D13S317 Stutter

D16S539 Stutter

D21S11 Stutter

D19S433 Stutter

vWA Stutter

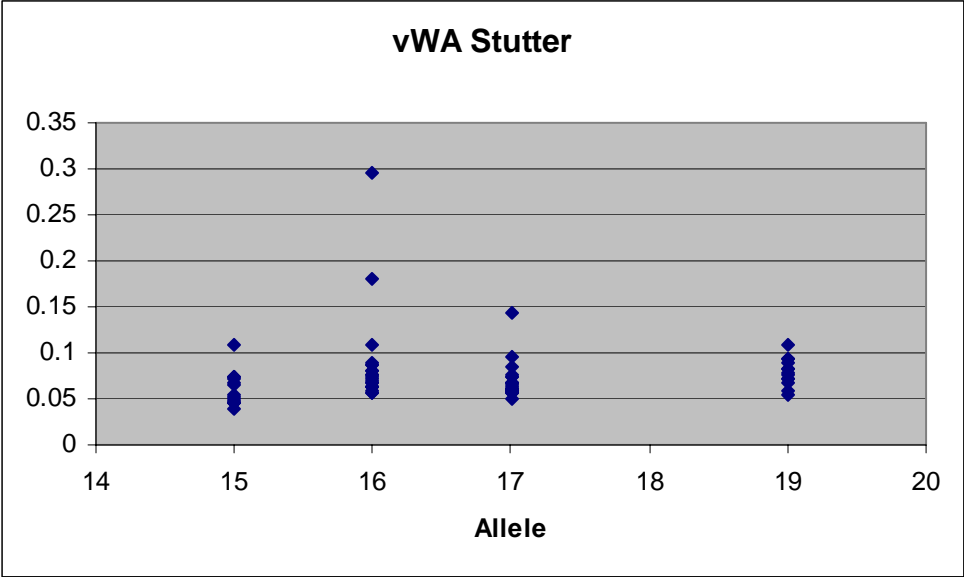TPOX Stutter

D18S51 Stutter
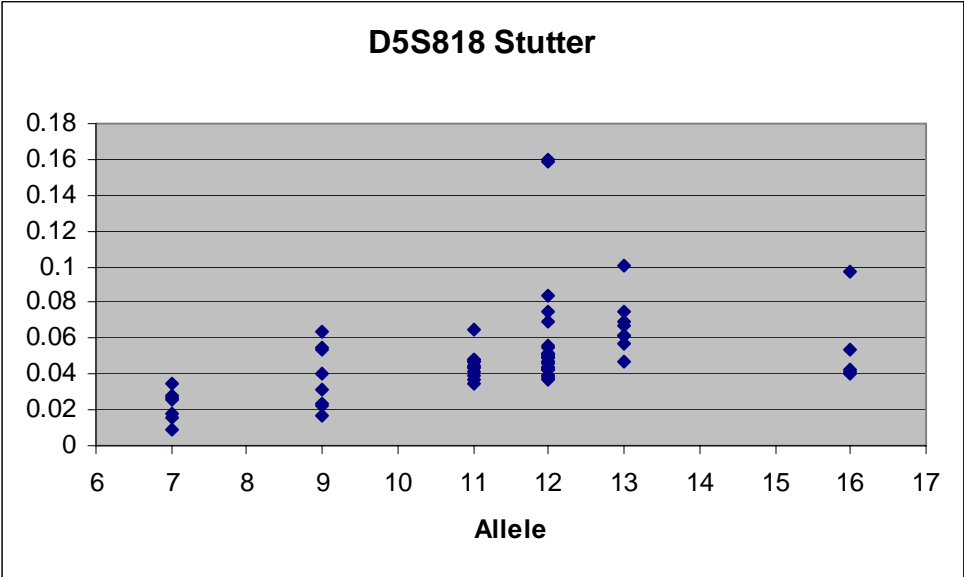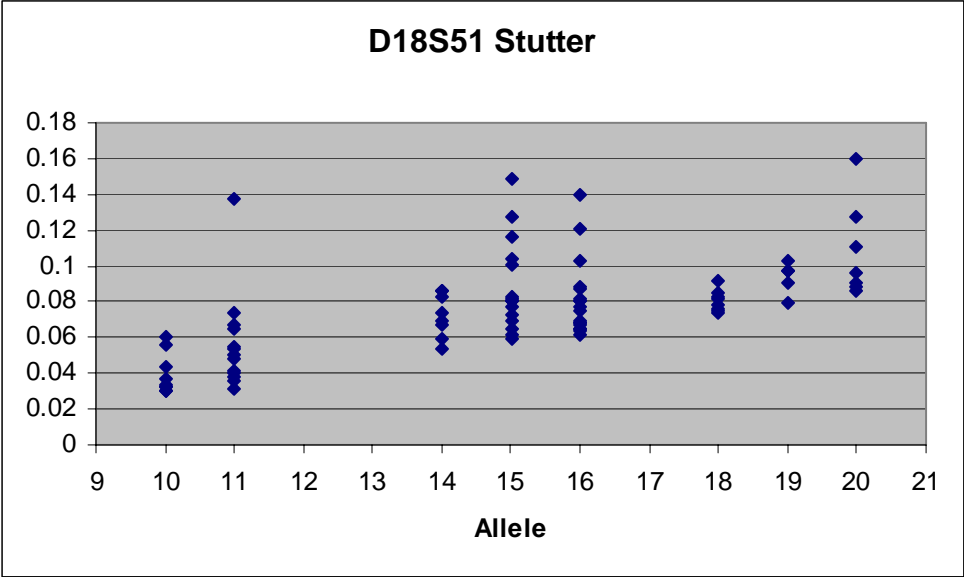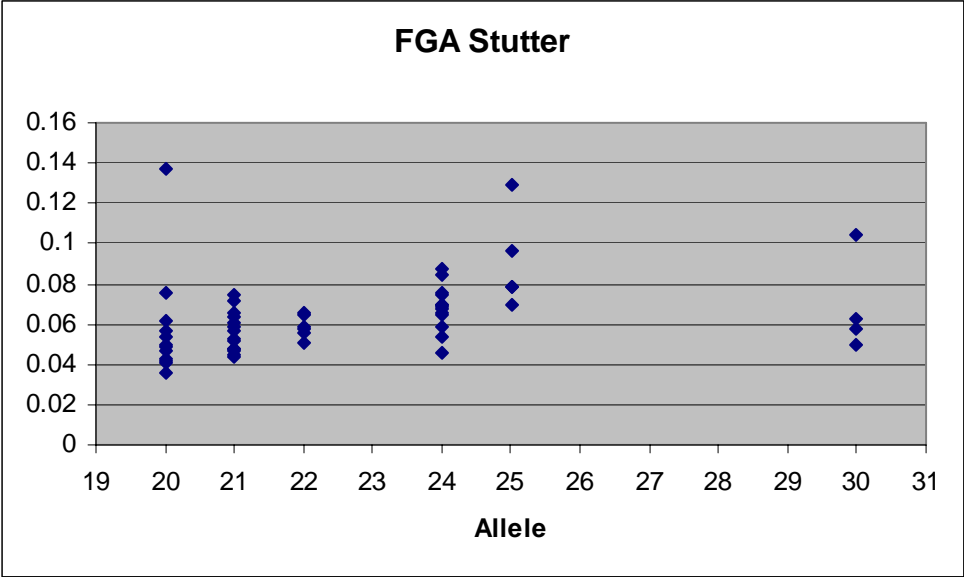

D5S818 Stutter

FGA Stutter

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